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(54) Title: CATALYTIC MOLECULES			
(57) Abstract			
<p>The present invention relates to DNazymes which are targeted against mRNA molecules encoding EGR-1 (also known as Egr-1 or NGFI-A). The present invention also relates to compositions including these DNazymes and to methods of treatment involving administration of the DNazymes.</p>			
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## CATALYTIC MOLECULES

### FIELD OF THE INVENTION

The present invention relates to DNazymes which are targeted against mRNA molecules encoding EGR-1 (also known as Egr-1 or NGFI-A). The present invention also relates to compositions including these DNazymes and to methods of treatment involving administration of the DNazymes.

### BACKGROUND OF THE INVENTION

#### Egr-1 expression in Smooth Muscle Cells

Smooth muscle cells (SMCs) are well recognized as a significant cellular component of atherosclerotic and post-angioplasty restenotic lesions (Stary et al, 1995; Holmes et al, 1984). SMC migration and proliferation are key events in the pathogenesis of these vascular disorders (Jackson & Schwartz, 1992; Libby et al, 1995). The promoter regions of many genes that encode mitogenic and migratory factors expressed by SMCs in these lesions (Evanko et al, 1998; Murry et al, 1996; Ueda et al, 1996; Tanizawa et al, 1996; Rekhter & Gordon, 1994; Hughes et al, 1993; Brogi et al, 1993; Wilcox et al, 1989; Wilcox et al, 1988) contain nucleotide (nt) recognition elements for the nuclear protein and transcription factor, Egr-1 (Khachigian & Collins, 1997; Khachigian et al, 1996). Egr-1 is not expressed in the unmanipulated artery wall, but is rapidly activated by mechanical injury (Khachigian et al, 1996; Silverman et al, 1997; Kim et al, 1995). It is also induced in vascular endothelial cells and/or SMCs exposed to fluid biomechanical forces (Khachigian et al, 1997; Sumpio et al, 1998) and multiple other pathophysiologically-relevant agonists (Delbridge & Khachigian, 1997).

#### DNazymes

In human gene therapy, antisense nucleic acid technology has been one of the major tools of choice to inactivate genes whose expression causes disease and is thus undesirable. The anti-sense approach employs a nucleic acid molecule that is complementary to, and thereby hybridizes with, an mRNA molecule encoding an undesirable gene. Such hybridization leads to the inhibition of gene expression.

Anti-sense technology suffers from certain drawbacks. Anti-sense hybridization results in the formation of a DNA/target mRNA heteroduplex. This heteroduplex serves as a substrate for RNase H-mediated degradation of the target mRNA component. Here, the DNA anti-sense molecule serves in a passive manner, in that it merely facilitates the required cleavage by endogenous RNase H enzyme. This dependence on RNase H confers limitations on the design of anti-sense molecules regarding their chemistry and ability to form stable heteroduplexes with their target mRNA's. Anti-sense DNA molecules also suffer from problems associated with non-specific activity and, at higher concentrations, even toxicity.

As an alternative to anti-sense molecules, catalytic nucleic acid molecules have shown promise as therapeutic agents for suppressing gene expression, and are widely discussed in the literature (Haseloff (1988); Breaker (1994); Koizumi (1989); Otsuka; Kashani-Sabet (1992); Raillard (1996); and Carmi (1996)). Thus, unlike a conventional anti-sense molecule, a catalytic nucleic acid molecule functions by actually cleaving its target mRNA molecule instead of merely binding to it. Catalytic nucleic acid molecules can only cleave a target nucleic acid sequence if that target sequence meets certain minimum requirements. The target sequence must be complementary to the hybridizing regions of the catalytic nucleic acid, and the target must contain a specific sequence at the site of cleavage.

Catalytic RNA molecules ("ribozymes") are well documented (Haseloff (1988); Symonds (1992); and Sun (1997)), and have been shown to be capable of cleaving both RNA (Haseloff (1988)) and DNA (Raillard (1996)) molecules. Indeed, the development of in vitro selection and evolution techniques has made it possible to obtain novel ribozymes against a known substrate, using either random variants of a known ribozyme or random-sequence RNA as a starting point (Pan (1992); Tsang (1994); and Breaker (1994)).

Ribozymes, however, are highly susceptible to enzymatic hydrolysis within the cells where they are intended to perform their function. This in turn limits their pharmaceutical applications.

Recently, a new class of catalytic molecules called "DNAzymes" was created (Breaker and Joyce (1995); Santoro (1997)). DNAzymes are single-stranded, and cleave both RNA (Breaker (1994); Santoro (1997)) and DNA (Carmi (1996)). A general model for the DNAzyme has been proposed, and is known as the "10-23" model. DNAzymes following the "10-23" model, also

referred to simply as "10-23 DNazymes", have a catalytic domain of 15 deoxyribonucleotides, flanked by two substrate-recognition domains of seven to nine deoxyribonucleotides each. In vitro analyses show that this type of DNzyme can effectively cleave its substrate RNA at purine:pyrimidine junctions under physiological conditions (Santoro (1997)).

5 DNazymes show promise as therapeutic agents. However, DNzyme success against a disease caused by the presence of a known mRNA molecule is not predictable. This unpredictability is due, in part, to two factors. First, certain mRNA secondary structures can impede a DNzyme's ability to bind  
10 to and cleave its target mRNA. Second, the uptake of a DNzyme by cells expressing the target mRNA may not be efficient enough to permit therapeutically meaningful results. For these reasons, merely knowing of a disease and its causative target mRNA sequence does not alone allow one to reasonably predict the therapeutic success of a DNzyme against that target  
15 mRNA, absent an inventive step.

#### SUMMARY OF THE INVENTION

Accordingly, in a first aspect the present invention provides a  
20 DNzyme which specifically cleaves EGR-1 mRNA, the DNzyme including  
(i) a catalytic domain which cleaves mRNA at a purine:pyrimidine cleavage site;  
(ii) a first binding domain contiguous with the 5' end of the catalytic domain; and  
25 (iii) a second binding domain contiguous with the 3' end of the catalytic domain,

wherein the binding domains are sufficiently complementary to two regions immediately flanking a purine:pyrimidine cleavage site within the region of EGR-1 mRNA corresponding to nucleotides 168 to 332 as shown in  
30 SEQ ID NO:1, such that the DNzyme cleaves the EGR-1 mRNA.

In a second aspect the present invention provides a pharmaceutical composition including a DNzyme according to the first aspect and a pharmaceutically acceptable carrier.

In a third aspect the present invention provides a method of inhibiting  
35 EGR-1 activity in cells which includes exposing the cells to a DNzyme according to the first aspect of the present invention.

In a fourth aspect the present invention provides a method of inhibiting proliferation or migration of cells in a subject which includes administering to the subject a prophylactically effective dose of a DNAzyme according to the first aspect of the present invention.

5        In a fifth aspect the present invention provides a method of treating a condition associated with cell proliferation or migration in a subject which includes administering to the subject a prophylactically effective dose of a DNAzyme according to the first aspect of the present invention.

10       In a sixth aspect the present invention provides an angioplastic stent for inhibiting the onset of restenosis, which comprises an angioplastic stent operably coated with a prophylactically effective dose of a DNAzyme according to the first aspect.

15       In a seventh aspect, the present invention provides a method for inhibiting the onset of restenosis in a subject undergoing angioplasty, which comprises topically administering a stent according to the fifth aspect to the subject at around the time of the angioplasty.

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1 Sequence of NGFI-A DNzyme (ED5), its scrambled control (ED5SCR) and 23 nt synthetic rat substrate. The translational start site is underlined.

Figure 2 NGFI-A DNzyme inhibits the induction of NGFI-A mRNA and protein by serum. Northern blot analysis was performed with 25  $\mu$ g of total RNA. The blot was stripped and reprobbed for  $\beta$ -Actin. Autoradiograms were analyzed by scanning densitometry and the ordinate axis is expressed as NGFI-A band intensity as a fraction of  $\beta$ -Actin band intensity. The mean and standard errors of the mean are indicated in the figure. Data is representative of 2 independent experiments. \* indicates  $P < 0.05$  (Student's paired t-test) as compared to control (FBS alone).

Figure 3 SMC proliferation is inhibited by NGFI-A DNzyme. a, Assessment of total cell numbers by Coulter counter. Growth-arrested SMCs that had been exposed to serum and/or DNzyme for 3 days were trypsinized followed by quantitation of the suspension. The sequence of AS2 is 5'-CTT GGC CGC TGC CAT-3' (SEQ ID NO: 20). b, Proportion of cells incorporating Trypan Blue after exposure to serum and/or DNzyme. Cells were stained incubated in 0.2% (w:v) Trypan Blue at 22 °C for 5 min prior to quantitation by hemocytometer in a blind manner. c, Effect of ED5 on pup SMC proliferation. Growth-arrested WKY12-22 cells exposed to serum and/or DNzyme for 3 days were resuspended and numbers were quantitated by Coulter counter. Data is representative of 2 independent experiments performed in triplicate. The mean and standard errors of the mean are indicated in the figure. \* indicates  $P < 0.05$  (Student's paired t-test) as compared to control (FBS alone).

Figure 4 NGFI-A DNzyme inhibition of neointima formation in the rat carotid artery. Neointimal and medial areas of 5 consecutive sections per rat (5 rats per group) taken at 250  $\mu$ m intervals from the point of ligation were determined digitally and expressed as a ratio per group. The mean and standard errors of the mean are indicated by the ordinate axis. \* denotes

$P < 0.05$  as compared to the Lig, Lig+Veh or Lig+Veh+ED5SCR groups using the Wilcoxon rank sum test for unpaired data. Lig denotes ligation, Veh denotes vehicle.

- 5    Figure 5    Selective inhibition of human smooth muscle cell proliferation by DzA.

Figure 6    Specific inhibition of porcine retinal smooth muscle cell proliferation by DzA.



## DETAILED DESCRIPTION OF THE INVENTION

Egr-1 (also known as NGFI-A and EGR-1) binds to the promoters of genes whose products influence cell movement and replication in the artery wall. Table 1 shows an alignment of the human EGR-1 cDNA sequence with the equivalent mouse (Egr-1) and rat (NGFI-A) sequences. The present inventors have now developed DNA-based enzymes that cut NGFI-A/Egr-1/EGR-1 RNA with high efficiency and specificity. The NGFI-A "DNAzyme" cleaved synthetic and *in vitro* transcribed NGFI-A RNA in a sequence-specific manner and inhibited production of NGFI-A in vascular smooth muscle cells without influencing levels of the related zinc finger protein, Sp1, or the immediate-early gene product, c-Fos. The DNAzyme blocked serum-inducible DNA synthesis in smooth muscle cells and attenuated total cell proliferation. The DNAzyme also inhibited the reparative response to mechanical injury, both in culture and in the rat carotid artery wall. These results indicate a necessary and sufficient role for NGFI-A/Egr-1/EGR-1 in vascular smooth muscle cell growth and provide the first demonstration of a DNAzyme targeted against NGFI-A/Egr-1/EGR-1 transcripts.

Accordingly, in a first aspect the present invention provides a DNAzyme which specifically cleaves EGR-1 mRNA, the DNAzyme including

- (i) a catalytic domain which cleaves mRNA at a purine:pyrimidine cleavage site;
- (ii) a first binding domain contiguous with the 5' end of the catalytic domain; and
- (iii) a second binding domain contiguous with the 3' end of the catalytic domain,

wherein the binding domains are sufficiently complementary to two regions immediately flanking a purine:pyrimidine cleavage site within the region of EGR-1 mRNA corresponding to nucleotides 168 to 332 as shown in SEQ ID NO:1, such that the DNAzyme cleaves the EGR-1 mRNA.

As used herein, "DNAzyme" means a DNA molecule that specifically recognizes and cleaves a distinct target nucleic acid sequence, which may be either DNA or RNA.

In a preferred embodiment of the first aspect of the present invention, the binding domains are complementary to the regions immediately flanking the cleavage site. It will be appreciated by those skilled in the art, however,

that strict complementarity may not be required for the DNAzyme to bind to and cleave the EGR-1 mRNA.

The catalytic domain of a DNAzyme of the present invention may be any suitable catalytic domain. Examples of suitable catalytic domains are described in *Santoro and Joyce, 1997* and US 5807718, the entire contents of which are incorporated herein by reference. In a preferred embodiment, the catalytic domain has the nucleotide sequence GGCTAGCTACAACGA (SEQ ID NO: 2).

Within the parameters of the present invention, the binding domain lengths (also referred to herein as "arm lengths") can be of any permutation, and can be the same or different. In a preferred embodiment, the binding domain lengths are at least 6 nucleotides. Preferably, both binding domains have a combined total length of at least 14 nucleotides. Various permutations in the length of the two binding domains, such as 7+7, 8+8 and 9+9, are envisioned. It is well established that the greater the binding domain length, the more tightly it will bind to its complementary mRNA sequence. Accordingly, in a more preferred embodiment, each domain is nine or more nucleotides in length.

Within the context of the present invention, preferred cleavage sites within the region of EGR-1 mRNA corresponding to nucleotides 168 to 332 are as follows:

- (i) the GU site corresponding to nucleotides 198-199;
- (ii) the GU site corresponding to nucleotides 200-201;
- (iii) the GU site corresponding to nucleotides 264-265;
- (iv) the AU site corresponding to nucleotides 271-272;
- (v) the AU site corresponding to nucleotides 301-302;
- (vi) the GU site corresponding to nucleotides 303-304; and
- (vii) the AU site corresponding to nucleotides 316-317.

In a further preferred embodiment, the DNAzyme has a sequence selected from:

- (i) 5'-caggggacaGGCTAGCTACAACGAacgttgcg (SEQ ID NO: 3)  
targets GU (nt 198, 199); arms hybridise to bp 189-207
- (ii) 5'-tgcaggggaGGCTAGCTACAACGAaccgttgcg (SEQ ID NO: 4)  
targets GU (nt 200, 201); arms hybridise to bp 191-209

- (iii) 5'-catcctggaGGCTAGCTACAACGAgagcaggct (SEQ ID NO: 5)  
targets GU (nt 264, 265); arms hybridise to bp 255-273
- 5 (iv) 5'-ccgcgccaGGCTAGCTACAACGAcctggacga (SEQ ID NO: 6)  
targets AU (nt 271, 272); arms hybridise to bp 262-280
- (v) 5'-ccgctccaGGCTAGCTACAACGAcccgacgt (SEQ ID NO: 7)  
targets AU (nt 271, 272); arms hybridise to bp 262-280
- 10 (vi) 5'-gcggggacaGGCTAGCTACAACGAcagctgcat (SEQ ID NO: 8)  
targets AU (nt 301, 302); arms hybridise to bp 292-310
- (vii) 5'-cagcggggaGGCTAGCTACAACGAatcagctgc (SEQ ID NO: 9).  
15 targets GU (nt 303, 304); arms hybridise to bp 294-312
- (viii) 5'-ggtcagagaGGCTAGCTACAACGActgcagcgg (SEQ ID NO: 10)  
targets AU (nt 316, 317); arms hybridise to bp 307-325.

20 In a particularly preferred embodiment, the DNAzyme targets the AU site corresponding to nucleotides 271-272 (ie. the translation start codon).

In a further preferred embodiment, the DNAzyme has the sequence:  
5'-ccgcgccaGGCTAGCTACAACGAcctggacga (SEQ ID NO: 6).

In applying DNAzyme-based treatments, it is preferable that the  
25 DNAzymes be as stable as possible against degradation in the intra-cellular milieu. One means of accomplishing this is by incorporating a 3'-3' inversion at one or more termini of the DNAzyme. More specifically, a 3'-3' inversion (also referred to herein simply as an "inversion") means the covalent  
30 phosphate bonding between the 3' carbons of the terminal nucleotide and its adjacent nucleotide. This type of bonding is opposed to the normal phosphate bonding between the 3' and 5' carbons of adjacent nucleotides, hence the term "inversion". Accordingly, in a preferred embodiment, the 3'-end nucleotide residue is inverted in the binding domain contiguous with the  
35 DNAzymes may contain modified nucleotides. Modified nucleotides

include, for example, N3'-P5' phosphoramidate linkages, and peptide-nucleic acid linkages. These are well known in the art.

In a particularly preferred embodiment, the DNAzyme includes an inverted T at the 3' position.

5 As will be appreciated by those skilled in the art, given that DNAzymes of the present invention cleave human EGR-1, similar DNAzymes can be produced to cleave the corresponding mRNA in other species, eg. rat (NGFI-A), mouse (Egr-1) etc. In a further aspect, the present invention provides such DNAzymes.

10 In a second aspect the present invention provides a pharmaceutical composition including a DNAzyme according to the first aspect and a pharmaceutically acceptable carrier.

In a third aspect the present invention provides a method of inhibiting EGR-1 activity in cells which includes exposing the cells to a DNAzyme  
15 according to the first aspect of the present invention.

In a fourth aspect the present invention provides a method of inhibiting proliferation or migration of cells in a subject which includes administering to the subject a prophylactically effective dose of a DNAzyme according to the first aspect of the present invention.

20 In a fifth aspect the present invention provides a method of treating a condition associated with cell proliferation or migration in a subject which includes administering to the subject a prophylactically effective dose of a DNAzyme according to the first aspect of the present invention.

In preferred embodiments of the third, fourth and fifth aspects of the  
25 present invention, the cells are vascular cells, particularly smooth muscle or endothelial cells. The cells may, however, be cells involved in neoplasia, such as tumour cells and the like.

Although the subject may be any animal or human, it is preferred that the subject is a human.

30 In a preferred embodiment, conditions associated with SMC proliferation (and migration) are selected from post-angioplasty restenosis, vein graft failure, transplant coronary disease and complications associated with atherosclerosis (cerebrovascular infarction (stroke), myocardial infarction (heart attack), hypertension or peripheral vascular disease  
35 (gangrene of the extremities).

Within the parameters of the fourth and fifth aspects of the present invention, any suitable mode of administration may be used to administer or deliver the DNAzyme.

In particular, delivery of the nucleic acid agents described may be  
5 achieved by one or more of the following methods:

- (a) Liposomes and liposome-protein conjugates and mixtures.
- (b) Using catheters to deliver intra-luminal formulations of the nucleic acid as a solution or in a complex with a liposome.
- (c) Catheter delivery to adventitial tissue as a solution or in a complex  
10 with a liposome.
- (d) Within a polymer formulation such as polyethylenimine (PEI) or pluronic gels or within ethylene vinyl acetate copolymer (EVAc). The polymer is preferably delivered intra-luminally.
- (e) The nucleic acid may be bound to a delivery agent such as a  
15 targetting moiety, or any suitable carrier such as a peptide or fatty acid molecule.
- (f) Within a viral-liposome complex, such as Sendai virus.
- (g) The nucleic acid may be delivered by a double angioplasty balloon device fixed to catheter.
- (h) The nucleic acid could be delivered on a specially prepared stent of  
20 the Schatz-Palmaz or derivative type. The stent could be coated with a polymer or agent impregnated with nucleic acid that allows controlled release of the molecules at the vessel wall.

In a preferred embodiment, the mode of administration is topical  
25 administration. Topical administration may be effected or performed using any of the various methods and delivery systems known to those skilled in the art. The topical administration can be performed, for example, via catheter and topical injection, and via coated stent as discussed below.

Pharmaceutical carriers for topical administration are well known in  
30 the art, as are methods for combining same with active agents to be delivered. The following delivery systems, which employ a number of routinely used carriers, are only representative of the many embodiments envisioned for administering the instant composition.

Topical delivery systems include, for example, gels and solutions, and  
35 can contain excipients such as solubilizers, permeation enhancers (e.g., fatty acids, fatty acid esters, fatty alcohols and amino acids), and hydrophilic

polymers (e.g., polycarbophil and polyvinylpyrrolidone). In the preferred embodiment, the pharmaceutically acceptable carrier is a liposome or a biodegradable polymer. Examples of agents which can be used in this invention include the following: (1) CellFectin, 1:1.5 (M/M) liposome formulation of the cationic lipid N,N',N'',N'''-tetramethyl-N,N',N'',N'''-tetrapalmitylspermine and dioleoyl phosphatidyl-ethanolamine (DOPE) (GIBCO BRL); (2) Cytofection GSV, 2:1 (M/M) liposome formulation of a cationic lipid and DOPE (Glen Research); (3) DOTAP (N-[1-(2,3-dioleoyloxy)-N,N,N-trimethyl-ammoniummethylsulfate) (Boehringer Mannheim); (4) Lipofectamine, 3:1 (M/M) liposome formulation of the polycationic lipid DOSPA and the neutral lipid DOPE (GIBCO BRL); (5) FuGENE<sup>6</sup> (Roche Molecular Biochemicals); (6) Superfect (Qiagen); and (7) Lipofectamine 2000 (Gibco-life Technologies).

Examples of suitable methods for topical administration of the DNAzymes of the present invention are described in Autieri et al. (1995), Simons et al. (1992), Morishita et al. (1993), Bennett and Schwartz (1995) and Frimerman et al. (1999).

Determining the prophylactically effective dose of the instant pharmaceutical composition can be done based on animal data using routine computational methods. In one embodiment, the prophylactically effective dose contains between about 0.1 mg and about 1 g of the instant DNAzyme. In another embodiment, the prophylactically effective dose contains between about 1 mg and about 100 mg of the instant DNAzyme. In a further embodiment, the prophylactically effective dose contains between about 10 mg and about 50 mg of the instant DNAzyme. In yet a further embodiment, the prophylactically effective dose contains about 25 mg of the instant DNAzyme.

In a sixth aspect the present invention provides an angioplastic stent for inhibiting the onset of restenosis, which comprises an angioplastic stent operably coated with a prophylactically effective dose of a DNAzyme according to the first aspect.

Angioplastic stents, also known by other terms such as "intravascular stents" or simple "stents", are well known in the art. They are routinely used to prevent vascular closure due to physical anomalies such as unwanted inward growth of vascular tissue due to surgical trauma. They often have a

tubular, expanding lattice-type structure appropriate for their function, and can optionally be biodegradable.

In this invention, the stent can be operably coated with the instant pharmaceutical composition using any suitable means known in the art.

5 Here, "operably coating" a stent means coating it in a way that permits the timely release of the pharmaceutical composition into the surrounding tissue to be treated once the coated stent is administered. Such coating methods, for example, can use the polymer polypyrrole.

10 In a seventh aspect, the present invention provides a method for inhibiting the onset of restenosis in a subject undergoing angioplasty, which comprises topically administering a stent according to the fifth aspect to the subject at around the time of the angioplasty.

As used herein, administration "at around the time of angioplasty" can be performed during the procedure, or immediately before or after the procedure. The administering can be performed according to known  
15 methods such as catheter delivery.

In order that the nature of the present invention may be more clearly understood, preferred forms thereof will now be described with reference to the following non-limiting Figures and Examples.

Table 1

Symbol comparison table: GenRunData:pileupdna.cmp CompCheck: 6876  
 5 GapWeight: 5.000  
 GapLengthWeight: 0.300  
 EGRlalign.msf MSF: 4388 Type: N April 7, 1998 12:07 Check: 5107  
 10 Name: mouseEGR1 Len: 4388 Check: 8340 Weight: 1.0 (SEQ ID NO:11)  
 Name: ratEGR1 Len: 4388 Check: 8587 Weight: 1.0 (SEQ ID NO:12)  
 Name: humanEGR1 Len: 4388 Check: 8180 Weight: 1.00 (SEQ ID NO:1)

NB. THIS IS RAT NGFI-A numbering

15	1	50
mouseEgr1	.....	.....
ratNGFIA	CCGCGGAGCC TCAGCTCTAC GCGCCTGGCG CCCTCCCTAC GCGGGCGTCC	
humanEGR1	.....	.....
20	51	100
mouseEGR1	.....	.....
ratEGR1	CCGACTCCCG CGCGCGTTCA GGCTCCGGGT TGGGAACCAA GGAGGGGGAG	
humanEGR1	.....	.....
25	101	150
mouseEGR1	.....	.....
ratEGR1	GGTGGGTGCG CCGACCCGGA AACACCATAT AAGGAGCAGG AAGGATCCCC	
humanEGR1	.....	.....
30	151	200
mouseEGR1	.....	.....
ratEGR1	CGCCGGAACA GACCTTATTT GGGCAGCGCC TTATATGGAG TGGCCCAATA	
humanEGR1	.....	.....
35	201	250
mouseEGR1	.....	.....
ratEGR1	TGGCCCTGCC GCTTCCGGCT CTGGGAGGAG GGGCGAACGG GGGTTGGGGC	
humanEGR1	.....	.....
40	251	300
mouseEGR1	.....	.....
ratEGR1	GGGGGCAAGC TGGGAAGTCC AGGAGCCTAG CCCGGGAGGC CACTGCCGCT	
humanEGR1	.....	.....
45	301	350
mouseEGR1	.....	.....
ratEGR1	GTTCCAATAC TAGGCTTTCC AGGAGCCTGA GCGCTCAGGG TGCCGGAGCC	
humanEGR1	.....	.....
50	351	400
mouseEGR1	.....	.....
ratEGR1	GGTCGCAGGG TGGGAAGCGCC CACCGCTCTT GGATGGGAGG TCTTCACGTC	
humanEGR1	.....	.....
55	401	450
mouseEGR1	.....	.....
ratEGR1	ACTCCGGGTC CTCCCGGTCG GTCCTTCCAT ATTAGGGCTT CCTGCTTCCC	
humanEGR1	.....	.....
60	451	500



	mouseEGR1	.....	.....	.....	.....	.....
	ratEGR1	ATATATGGCC	ATGTACGTCA	CGGCGGAGGC	GGGCCCGTGC	TGTTTCAGAC
	humanEGR1	.....	.....	.....	.....	.....
5		501				550
	mouseEGR1	.....	.....	.....	.....	.....
	ratEGR1	CCTTGAAATA	GAGGCCGATT	CGGGGAGTCG	CGAGAGATCC	CAGCGCGCAG
	humanEGR1	.....	.....	.....	.....	....CCGCAG
10		551				600
	mouseEGR1	.....GGGGA	GCCGCCGCCG	CGATTGCGCG	CCGCCGCCAG	CTTCGCCCGC
	ratEGR1	AACTTGGGGA	GCCGCCGCCG	CGATTGCGCG	CCGCCGCCAG	CTTCGCCCGC
	humanEGR1	AACTTGGGGA	GCCGCCGCCG	CCATCCGCCG	CCGCAGCCAG	CTTCGCCCGC
15		601				650
	mouseEGR1	CGCAAGATCG	GCCCCTGCCC	CAGCCTCCGC	GGCAGCCCTG	CGTCCACCAC
	ratEGR1	CGCAAGATCG	GCCCCTGCCC	CAGCCTCCGC	GGCAGCCCTG	CGTCCACCAC
	humanEGR1	CGCAGGACCG	GCCCCTGCCC	CAGCCTCCGC	AGCCGCGGCG	CGTCCACGCC
20		651				700
	mouseEGR1	GGGCCGCGGC	TACCGCCAGC	CTGGGGGCCC	ACCTACACTC	CCCGCAGTGT
	ratEGR1	GGGCCGCGGC	CACCGCCAGC	CTGGGGGCCC	ACCTACACTC	CCCGCAGTGT
	humanEGR1	CGCCCGCGCC	CAGGGCGAGT	CGGGGTCGCC	GCCTGCACGC	TTCTCAGTGT
25		701				750
	mouseEGR1	GCCCCTGAC	CCCGCATGTA	ACCCGGCCAA	CCCCCGGCGA	GTGTGCCCTC
	ratEGR1	GCCCCTGAC	CCCGCATGTA	ACCCGGCCAA	CATCCGGCGA	GTGTGCCCTC
	humanEGR1	TCCCC.GCGC	CCCGCATGTA	ACCCGGCCAG	GCCCCCGCAA	CGGTGTCCCC
30		751				800
	mouseEGR1	AGTAGCTTCG	GCCCCGGGCT	GCGCCCACC.	.ACCCAACAT	CAGTTCTCCA
	ratEGR1	AGTAGCTTCG	GCCCCGGGCT	GCGCCCACC.	.ACCCAACAT	CAGCTCTCCA
	humanEGR1	TGCAGCTCCA	GCCCCGGGCT	GCACCCCCC	GCCCCGACAC	CAGCTCTCCA
35		801				850
	mouseEGR1	GCTCGCTGGT	CCGGGATGGC	AGCGGCCAAG	GCCGAGATGC	AATTGATGTC
	ratEGR1	GCTCGCACGT	CCGGGATGGC	AGCGGCCAAG	GCCGAGATGC	AATTGATGTC
	humanEGR1	GCCTGCTCGT	CCAGGATGGC	CGCGGCCAAG	GCCGAGATGC	AGCTGATGTC
40		ED5 (rat) arms hybridise to bp 807-825 in rat sequ				
		hED5(hum) arms hybridise to bp 262-280 in hum sequ				
		851				900
	mouseEGR1	TCCGCTGCAG	ATCTCTGACC	CGTTCGGGTC	CTTTCCTCAC	TCACCCACCA
45	ratEGR1	TCCGCTGCAG	ATCTCTGACC	CGTTCGGGTC	CTTTCCTCAC	TCACCCACCA
	humanEGR1	CCCGCTGCAG	ATCTCTGACC	CGTTCGGATC	CTTTCCTCAC	TCGCCCACCA
		901				950
	mouseEGR1	TGGACAACCTA	CCCCAACTG	GAGGAGATGA	TGCTGCTGAG	CAACGGGGCT
50	ratEGR1	TGGACAACCTA	CCCCAACTG	GAGGAGATGA	TGCTGCTGAG	CAACGGGGCT
	humanEGR1	TGGACAACCTA	CCCTAAGCTG	GAGGAGATGA	TGCTGCTGAG	CAACGGGGCT
		951				1000
	mouseEGR1	CCCCAGTTCC	TCGGTGCTGC	CGGAACCCCA	GAGGGCAGCG	GCGGTAAT..
55	ratEGR1	CCCCAGTTCC	TCGGTGCTGC	CGGAACCCCA	GAGGGCAGCG	GCGGCAATAA
	humanEGR1	CCCCAGTTCC	TCGGCGCCGC	CGGGGCCCCA	GAGGGCAGCG	GCAGCAACAG
		1001				1050
	mouseEGR1	.....AGC	AGCAGCAGCA	CCAGCAGCGG	GGGCGGTGGT	GGGGGCGGCA
60	ratEGR1	CAGCAGCAGC	AGCAGCAGCA	GCAGCAGCGG	GGGCGGTGGT	GGGGGCGGCA

	humanEGR1	CAGCAGCAGC	AGCAGCGGGG	GCGGTGGAGG	CGGCGGGGGC	GGCAGCAACA
		1051				1100
5	mouseEGR1	GCAACAGCGG	CAGCAGCGCC	TTCAATCCTC	AAGGGGAGCC	GAGCGAACAA
	ratEGR1	GCAACAGCGG	CAGCAGCGCT	TTCAATCCTC	AAGGGGAGCC	GAGCGAACAA
	humanEGR1	GCAGCAGCAG	CAGCAGCACC	TTCAACCCTC	AGGCGGACAC	GGGCGAGCAG
		1101				1150
10	mouseEGR1	CCCTATGAGC	ACCTGACCAC	AG...AGTCC	TTTTCTGACA	TCGCTCTGAA
	ratEGR1	CCCTACGAGC	ACCTGACCAC	AGGTAAGCGG	TGGTCTGCGC	CGAGGCTGAA
	humanEGR1	CCCTACGAGC	ACCTGACCAC	AG...AGTCT	TTTCTGACA	TCTCTCTGAA
		1151				1200
15	mouseEGR1	TAATGAGAAG	GCGATGGTGG	AGACGAGTTA	TCCCAGCCAA	ACGACTCGGT
	ratEGR1	TCCCCCTTCG	TGACTACCCT	AACGTCCAGT	CCTTTGCAGC	ACGGACCTGC
	humanEGR1	CAACGAGAAG	GTGCTGGTGG	AGACCAGTTA	CCCCAGCCAA	ACCACTCGAC
		1201				1250
20	mouseEGR1	TGCCCTCCCAT	CACCTATACT	GGCCGCTTCT	CCCTGGAGCC	CGCACCCCAAC
	ratEGR1	ATCTAGATCT	TAGGGACGGG	ATTGGGATTT	CCCTCTATTC	..CACACAGC
	humanEGR1	TGCCCCCAT	CACCTATACT	GGCCGCTTTT	CCCTGGAGCC	TGCACCCCAAC
		1251				1300
25	mouseEGR1	AGTGGCAACA	CTTTGTGGCC	TGAACCCCTT	TTCAGCCTAG	TCAGTGGCCT
	ratEGR1	TCCAGGGACT	TGTGTAGAG	GGATGTCTGG	GGACCCCA	ACCCTCCATC
	humanEGR1	AGTGGCAACA	CCTTGTGGCC	CGAGCCCTC	TTCAGCTTGG	TCAGTGGCCT
		1301				1350
30	mouseEGR1	CGTGAGCATG	ACCAATCCTC	CGACCTCTTC	ATCCTCGGCG	CCTTCTCCAG
	ratEGR1	CTTGCGGGTG	CGCGGAGGGC	AGACCGTTTG	TTTTGGATGG	AGAAGCTCAAG
	humanEGR1	AGTGAGCATG	ACCAACCCAC	CGGCCTCCTC	GTCTCTAGCA	CCATCTCCAG
		1351				1400
35	mouseEGR1	CTGCTTCATC	GTCTTCTCT	GCCTCCAGA	GCCCGCCCT	GAGCTGTGCC
	ratEGR1	TTGCGTGGGT	GGCT.....	.....GGAGT	GGGGGAGGGT	TTGTTTTGAT
	humanEGR1	CGGCCTCCTC	CGC...CTCC	GCCTCCAGA	GCCACCCCT	GAGCTGCGCA
		1401				1450
40	mouseEGR1	GTGCCGTCCA	ACGACAGCAG	TCCCATCTAC	TCGGCTGCGC	CCACCTTTCC
	ratEGR1	GAGCAGGGTT	GC....CCCC	TCCCCCGCGC	GCGTTGTCGC	GAGCCTTGTT
	humanEGR1	GTGCCATCCA	ACGACAGCAG	TCCCATTTAC	TCAGCGGCAC	CCACCTTTCC
		1451				1500
45	mouseEGR1	TACTCCCAAC	ACTGACATTT	TTCTGAGCC	CCAAAGCCAG	GCCTTTCTG
	ratEGR1	TGCAGCTTGT	TCCCAAGGAA	GGGCTGAAAT	CTGTCAACAG	GGATGTCCCG
	humanEGR1	CACGCCGAAC	ACTGACATTT	TTCTGAGCC	ACAAAGCCAG	GCCTTTCCCG
		1501				1550
50	mouseEGR1	GCTCGGCAGG	CACAGCCTTG	CAGTACCCGC	CTCCTGCCTA	CCCTGCCACC
	ratEGR1	CCGCCCAGGG	TAGGGGCGCG	CATTAGCTGT	GGCC..ACTAG	GGTGTGGCG
	humanEGR1	GCTCGGCAGG	GACAGCGCTC	CAGTACCCGC	CTCCTGCCTA	CCCTGCCGCC
		1551				1600
55	mouseEGR1	AAAGGTGGTT	TCCAGGTTCC	CATGATCCCT	GACTATCTGT	TTCCACAACA
	ratEGR1	GGATTCCCTC	ACCCCGGACG	CCTGCTGCGG	AGCGCTCTCA	GAGCTGCAGT
	humanEGR1	AAGGGTGGCT	TCCAGGTTCC	CATGATCCCC	GACTACCTGT	TTCCACAGCA
		1601				1650
60	mouseEGR1	ACAGGGAGAC	CTGAGCCTGG	GCACCCAGA	CCAGAAGCCC	TTCCAGGGTC
	ratEGR1	AGAGGGGGAT	TCTCTGTTTG	CGTCAGCTGT	CGAAATGGCT	CT.....GC

	humanEGR1	GCAGGGGGAT	CTGGGCCTGG	GCACCCAGAG	CCAGAAGCCC	TTCCAGGGCC
		1651				1700
5	mouseEGR1	TGGAGAACCG	TACCCAGCAG	CCTTCGCTCA	CTCCACTATC	CACTATTAAA
	ratEGR1	CACTGGAGCA	GGTCCAGGAA	CATTGCAATC	TGCTGCTATC	AATTATTAAC
	humanEGR1	TGGAGAGCCG	CACCCAGCAG	CCTTCGCTAA	CCCCTCTGTC	TACTATTAAG
		1701				1750
10	mouseEGR1	GCCTTCGCCA	CTCAGTCGGG	CTCCCAGGAC	TTAAAG....	...GCTCTTA
	ratEGR1	CACATCGAGA	GTCAGTGGTA	GCCGGGCGAC	CTCTTGCCCTG	GCCGCTTCGG
	humanEGR1	GCCTTTGCCA	CTCAGTCGGG	CTCCCAGGAC	CTGAAG....	...GCCCTCA
		1751				1800
15	mouseEGR1	ATACCACCTA	CCAATCCCAG	CTCATCA..A	ACCCAGCCGC	ATGCGCAAGT
	ratEGR1	CTCTCATCGT	CCAGTGATTG	CTCTCCAGTA	ACCAGGCCTC	TCTGTTCTCT
	humanEGR1	ATACCAGCTA	CCAGTCCCAG	CTCATCA..A	ACCCAGCCGC	ATGCGCAAGT
		1801				1850
20	mouseEGR1	ACCCCAACCG	GCCCAGCAAG	ACACCCCCC	ATGAACGCCC	ATATGCTTGC
	ratEGR1	TTCTTGCCAG	AGTCCTTTTC	TGACATCGCT	CTGAATAACG	AGAAG..GCG
	humanEGR1	ATCCCAACCG	GCCCAGCAAG	ACGCCCCC	ACGAACGCCC	TTACGCTTGC
		1851				1900
25	mouseEGR1	CCTGTGCGAGT	CCTGCGATCG	CCGCTTTTCT	CGCTCGGATG	AGCTTACCCG
	ratEGR1	CTGGTGGAGA	CAAGTTATCC	CAGCCAACT	ACCCGGTTGC	CTCCCATCAC
	humanEGR1	CCAGTGGAGT	CCTGTGATCG	CCGCTTCTCC	CGCTCCGACG	AGCTCACCCG
		1901				1950
30	mouseEGR1	CCATATCCGC	ATCCACACAG	GCCAGAAGCC	CTTCCAGTGT	CGAATCTGCA
	ratEGR1	CTATACTGGC	CGCTTCTCCC	TGGAGCCTGC	ACCCAACAGT	GCCAACACTT
	humanEGR1	CCACATCCGC	ATCCACACAG	GCCAGAAGCC	CTTCCAGTGC	CGCATCTGCA
		1951				2000
35	mouseEGR1	TGCGTAACTT	CAGTCGTAGT	GACCACCTTA	CCACCCACAT	CCGCACCCAC
	ratEGR1	TGTGGCCTGA	ACCCCTTTTC	AGCCTAGTCA	GTGGCCTTGT	GAGCATGACC
	humanEGR1	TGCGCAACTT	CAGCCGCAGC	GACCACCTCA	CCACCCACAT	CCGCACCCAC
		2001				2050
40	mouseEGR1	ACAGGCGAGA	AGCCTTTTGC	CTGTGACATT	TGTGGGAGGA	AGTTTGCCAG
	ratEGR1	AACCCCTCAA	CCTCTTCATC	CTCAGCGCCT	TCTCCAGCTG	CTTCATCGTC
	humanEGR1	ACAGGCGAAA	AGCCTTTCGC	CTGCGACATC	TGTGGAAGAA	AGTTTGCCAG
		2051				2100
45	mouseEGR1	GAGTGATGAA	CGCAAGAGGC	ATACCAAAAT	CCATTTAAGA	CAGAAGGACA
	ratEGR1	TTCTCTGCCC	TCCCAGAGCC	CACCCCTGAG	CTGTGCCGTG	CCGTCCAACG
	humanEGR1	GAGCGATGAA	CGCAAGAGGC	ATACCAAGAT	CCACTTGCGG	CAGAAGGACA
		2101				2150
50	mouseEGR1	AGAAAGCAGA	CAAAAGTGTG	GTGGCCTCCC	CGGCTGC...	...CTCTTCACT
	ratEGR1	ACAGCAGTCC	CATTTACTCA	GCTGCACCCA	CCTTTCTTAC	TCCCAACACT
	humanEGR1	AGAAAGCAGA	CAAAAGTGTT	GTGGCCTCTT	CGGCCACCTC	CTCTCTCTCT
		2151				2200
55	mouseEGR1	.....	.....	CTCTTCTTAC	CCATCCCCAG	TGGCTACCTC
	ratEGR1	.....	.....	GACATTTTTC	CTGAGCCCCA	AAGCCAGGCC
	humanEGR1	TCCTACCCGT	CCCCGGTTGC	TACCTCTTAC	CCGTCCCCGG	TTACTACCTC
		2201				2250
60	mouseEGR1	CTACCCATCC	CCTGCCACCA	CCTCATTCCC	ATCCCCTGTG	CCCACTTCCT
	ratEGR1	TTTCTGGCT	CTGCAGGCAC	AGCCTTGCG	TACCCGCTC	CTGCCTACCC

	humanEGR1	TTATCCATCC	CCGGCCACCA	CCTCATACCC	ATCCCCTGTG	CCCACCTCCT	
		2251				2300	
5	mouseEGR1	ACTCCTCTCC	TGGCTCCTCC	ACCTACCCAT	CTCCTGCGCA	CAGTGGCTTC	
	ratEGR1	TGCCACCAAG	GGTGGTTTCC	AGGTTCCTAT	GATCCCTGAC	TATCTGTTTC	
	humanEGR1	TCTCCTCTCC	CGGCTCCTCG	ACCTACCCAT	CCCCTGTGCA	CAGTGGCTTC	
		2301				2350	
10	mouseEGR1	CCGTCGCCGT	CAGTGGCCAC	CACCTTTGCC	TCCGTTCC..	.....	
	ratEGR1	CACAACAACA	GGGAGACCTG	AGCCTGGGCA	CCCCAGACCA	GAAGCCCTTC	
	humanEGR1	CCCTCCCCGT	CGGTGGCCAC	CACGTACTCC	TCTGTTCC..	.....	
		2351				2400	
15	mouseEGR1	....ACCTGC	TTTCCCCACC	CAGGTCAGCA	GCTTCCCGTC	TGCGGGCGTC	
	ratEGR1	CAGGGTCTGG	AGAACCCTAC	CCAGCAGCCT	TCGCTCACTC	CACTATCCAC	
	humanEGR1	....CCTGC	TTTCCCGGCC	CAGGTCAGCA	GCTTCCCTTC	CTCAGCTGTC	
		2401				2450	
20	mouseEGR1	AGCAGCTCCT	TCAGCACCTC	AACTGGTCTT	TCAGACATGA	CAGCGACCTT	
	ratEGR1	TATCAAAGCC	TTGCGCACTC	AGTCGGGCTC	CCAGGACTTA	AAGGCTCTTA	
	humanEGR1	ACCAACTCCT	TCAGCGCCTC	CACAGGGCTT	TCGGACATGA	CAGCAACCTT	
		2451				2500	
25	mouseEGR1	TTCTCCCAGG	ACAATTGAAA	TTTGCTAAAG	GGA.....	ATAAAAG..	
	ratEGR1	ATAACACCTA	CCAGTCCCAA	CTCATCAAAAC	CCAGCCGCAT	GCGCAAGT..	
	humanEGR1	TTCTCCCAGG	ACAATTGAAA	TTTGCTAAAG	GGAAAGGGGA	AAGAAAGGGA	
		2501				2550	
30	mouseEGR1	.AAAGCAAAG	GGAGAGGCAG	GAAAGACATA	AAAGCA...C	AGGAGGGGAG	
	ratEGR1	.ACCCCAACC	GGCCAGCAAP	GACACCCCCC	CATGAACGCC	CGTATGCTTG	
	humanEGR1	AAAGGGAGAA	AAAGAAACAC	AAGAGACTTA	AAGGACAGGA	GGAGGAGATG	
		2551				2600	
35	mouseEGR1	AGATGGCCGC	AAGAGGGGCC	ACCTCTTAGG	TCAGATGGAA	GATCTCAGAG	
	ratEGR1	CCCTGTTGAG	TCCTGCGATC	GCCGCTTTTC	TCGCTCGGAT	GAGCTTACAC	
	humanEGR1	GCCATAGGAG	AGGAGGGTT.	.CCTCTTAGG	TCAGATGGAG	GTCTCAGAG	
		2601				2650	
40	mouseEGR1	CCAAGTCCTT	CTACTCACGA	GTA..GAAGG	ACCCTTGGCC	AACAGCCCTT	
	ratEGR1	GCCACATCCG	CATCCATACA	GGC..CAGAA	GCCCTTCCAG	TGTGGAATCT	
	humanEGR1	CCAAGTCCTC	CCTCTCTACT	GGAGTGGGAG	GTCTATTGGC	CAACAATCCT	
		2651				2700	
45	mouseEGR1	TCACTTACCA	TCCCTGCCTC	CCCCGTCTG	TTCCCTTTGA	CTTCAGCTGC	
	ratEGR1	GCATGCGTAA	TTTCAGTCGT	AGTGACCACC	TTACCACCCA	CATCCGCACC	
	humanEGR1	TTCTGCCCAC	TTCCCTTCC	CCAATTACTA	TTCCCTTTGA	CTTCAGCTGC	
		2701				2750	
50	mouseEGR1	CTGAAACAGC	CATGTCCAAG	TTCTTCACCT	CTATCCAAAG	GACTTGATTT	
	ratEGR1	C..ACACAGG	CGAGAAGCCT	TTGCCTGTG	ACATTTGTGG	GAGAAAGTTT	
	humanEGR1	CTGAAACAGC	CATGTCCAAG	TTCTTCACCT	CTATCCAAAG	AACTTGATTT	
		2751				2800	
55	mouseEGR1	GCATGG....	..TATTGGAT	AAATCATTTT	AGTATCCTCT	.....	
	ratEGR1	GCCAGGAGTG	ATGAACGCAA	GAGGCATACC	AAAATCCACT	TAAGACAGAA	
	humanEGR1	GCATGGA...	..TTTGGAT	AAATCATTTT	AGTATCATCT	.....	
		2801				2850	
60	mouseEGR1	.....CCATC	ACATGCCTGG	CCCTTGCTCC	CTTCAGCGCT	AGACCATCAA	
	ratEGR1	GGACAAGAAA	GCAGACAAAA	GTGTCGTGGC	CTCCTCAGCT	GCCTCTTCCC	

	humanEGR1	....CCATCA	TATGCCTGAC	CCCTTGCTCC	CTTCAATGCT	AGAAAATCGA	
		2851					2900
5	mouseEGR1	GTTGGCATAA	AGAAAAAAA	ATGGGTTTGG	GCCCTCAGAA	CCCTGCCCTG	
	ratEGR1	TCTCTTCCTA	CCCATCCCCA	GTGGCTACCT	CCTACCCATC	CCCCGCCACC	
	humanEGR1	GTTGGC....	.....AAAAT	GGGGTTTGGG	CCCCTCAGAG	CCCTGCCCTG	
		2901					2950
10	mouseEGR1	CATCTTTGTA	CAGCATCTGT	GCCATGGATT	TTGTTTTCTT	TGGGGTATTC	
	ratEGR1	ACCTCATTTC	CATCCCCAGT	GCCCACCTCT	TACTCCTCTC	CGGGCTCCTC	
	humanEGR1	CACCCTTGTA	CAGTGTCTGT	GCCATGGATT	TCGTTTTTCT	TGGGGTACTC	
		2951					3000
15	mouseEGR1	TTGATGTGAA	GATAATTTGC	ATACT.....	.CTATTGTAT	TATTTGGAGT	
	ratEGR1	TACCTACCCG	TCTCCTGCAC	ACAGTGGCTT	CCCATCGCCC	TCGGTGGCCA	
	humanEGR1	TTGATGTGAA	GATAATTTGC	ATATT.....	.CTATTGTAT	TATTTGGAGT	
		3001					3050
20	mouseEGR1	TAAATCCTCA	CTTTGGGG..	GAGGGGGGAG	CAAAGCCAAG	CAAACCAATG	
	ratEGR1	CCACCTATGC	CTCCGTCC..	CACCTGCTTT	CCCTGCCCAG	GTCAGACCTT	
	humanEGR1	TAGGTCTCTA	CTTGGGGGAA	AAAAAAAAAA	AAAAGCCAAG	CAAACCAATG	
		3051					3100
25	mouseEGR1	ATGATCCTCT	ATTTTGTGAT	GA CTCTGCTG	TGACATTA..	.....	
	ratEGR1	TCCAGTCTGC	AGGGGTCAGC	AACTCCTTCA	GCACCTCAAC	GGGTCTTTCA	
	humanEGR1	GTGATCCTCT	ATTTTGTGAT	GATGCTGTGA	CAATA.....	.....	
		3101					3150
30	mouseEGR1	.GGTTTGAAG	CATTTTTTTT	TTCAAGCAGC	AGTCCTAGGT	ATTAACTGGA	
	ratEGR1	GACATGACAG	CAACCTTTTC	TCCTAGGACA	ATTGAAATTT	GCTAAAGGGA	
	humanEGR1	...AGTTTGA	ACCTTTTTTT	TTGAAACAGC	AGTCCCAG..	..TATCTCA	
		3151					3200
35	mouseEGR1	..GCATGTGT	CAGAGTGTG	TTCCGTAAAT	TTGTAAATA	CTGGCTCGAC	
	ratEGR1	ATGAAAGAGA	GCAAAGGGAG	GGGAGCGCGA	GAGACAATAA	AGGACAGGAG	
	humanEGR1	GAGCATGTGT	CAGAGTGTG	TTCCGTAAAC	CTTTTGTAA	ATACTGCTTG	
		3201					3250
40	mouseEGR1	.TGTAACCTCT	CACATGTGAC	AAAGTATGGT	TTGTTTGGTT	GGGTTTGT	
	ratEGR1	.GGAAGAAAT	GGCCCGCAAAG	AGGGGCTGCC	TCTTAGGTCA	GATGGAAGAT	
	humanEGR1	ACCGTACTCT	CACATGTGGC	AAAATATGGT	TTGGTTTTTC	TTTTTTTTTT	
		3251					3300
45	mouseEGR1	TTTGAGAATT	TTTTTGCCCG	TCCCTTTGGT	TTCAAAAGTT	TCACGTCTTG	
	ratEGR1	CTCAGAGCCA	AGTCCTTCTA	GTCAGTAGAA	GGCCCGTTGG	CCACCAGCCC	
	humanEGR1	TTGAAAGTGT	TTTTTCTTCG	TCCTTTTGGT	TTAAAAAGTT	TCACGTCTTG	
		3301					3350
50	mouseEGR1	GTGCCTTTTG	TGTGACACGC	CTT.CCGATG	GCTTGACATG	CGCA.....	
	ratEGR1	TTTCACTTAG	CGTCCCTGCC	CTC.CCCAGT	CCCGGCTCCT	TTGACTTCAG	
	humanEGR1	GTGCCTTTTG	TGTGATGCCC	CTTGCTGATG	GCTTGACATG	TGCAAT....	
		3351					3400
55	mouseEGR1	...GATGTGA	GGGACACGCT	CACCTTAGCC	TTAA...GGG	GGTAGGAGTG	
	ratEGR1	CTGCCTGAAA	CAGCCACGTC	CAAGTTCTTC	ACCT...CTA	TCCAAAGGAC	
	humanEGR1	.....TGTGA	GGGACATGCT	CACCTCTAGC	CTTAAGGGGG	GCAGGGAGTG	
		3401					3450
60	mouseEGR1	ATGTGTTGGG	GGAGGCTTGA	GAGCAAAAAC	GAGGAAGAGG	GCTGAGCTGA	
	ratEGR1	TTGATTGCA	TGGTATTGGA	TAAACCATT	CAGCATCATC	TCCACCACAT	

	humanEGR1	ATGATTGGG	GGAGGCTTG	GGAGCAAAAT	AAGGAAGAGG	GCTGAGCTGA	
		3451				3500	
5	mouseEGR1	GCTTCGGTC	TCCAGAATGT	AAGAAGAAAA	AATTTAAACA	AAAATCTGAA	
	ratEGR1	GCCTGGCCCT	TGCTCCCTTC	AGCACTAGAA	CATCAAGTTG	GCTGAAAAAA	
	humanEGR1	GCTTCGGTTC	TCCAGAATGT	AAGAAAACAA	AATCTAAAC	AAAATCTGAA	
		3501				3550	
10	mouseEGR1	CTCTCAAAAG	TCTATTTTTC	TAAACTGAAA	ATGTAAATTT	ATACATCTAT	
	ratEGR1	AAAATGGGTC	TGGGCCCTCA	GAACCCTGCC	CTGTATCTTT	GTACA.....	
	humanEGR1	CTCTCAAAAG	TCTATTTTTC	TAA..CTGAAA	ATGTAAATTT	ATAAATATAT	
		3551				3600	
15	mouseEGR1	TCAGGAGTTG	GAGTGTGTG	GTTACCTACT	GAGTAGGCTG	CAGTTTTTGT	
	ratEGR1	GCATCTGTGC	CATGGATTTT	GTTTCCTTG	GGGTATTCTT	GATGTGAAGA	
	humanEGR1	TCAGGAGTTG	GAATGTGTG	GTTACCTACT	GAGTAGGCGG	CGATTTTTGT	
		3601				3650	
20	mouseEGR1	ATGTTATGAA	CATGAAGTTC	ATTATTTTGT	GGTTTTATTT	TACTTTGTAC	
	ratEGR1	TAATTTGCAT	ACTCTATTGT	ACTATTTGGA	GTTAAATTCT	CACTTTGGGG	
	humanEGR1	ATGTTATGAA	CATGCAGTTC	ATTATTTTGT	GGTTCTATTT	TACTTTGTAC	
		3651				3700	
25	mouseEGR1	TTGTGTTTGC	TTAAACAAAG	TAACCTGTTT	GGCTTATAAA	CACATTGAAT	
	ratEGR1	GAGGGGGAGC	AAAGCCAAGC	AAACCAATGG	TGATCCTCTA	TTTTGTGATG	
	humanEGR1	TTGTGTTTGC	TTAAACAAAG	TGA..CTGTTT	GGCTTATAAA	CACATTGAAT	
		3701				3750	
30	mouseEGR1	GCGCTCTATT	GCCCATGG..	..GATATGTG	GTGTGTATCC	TTCAGAAAAA	
	ratEGR1	ATCCTGCTGT	GACATTAGGT	TTGAAACTTT	TTTTTTTTTT	TGAAGCAGCA	
	humanEGR1	GCGCTTTATT	GCCCATGG..	..GATATGTG	GTGTATATCC	TTCCAAAAAA	
		3751				3800	
35	mouseEGR1	TTAAAAGGAA	AAAT.....	.....	.....	.....	
	ratEGR1	GTCCTAGGTA	TTAACTGGAG	CATGTGTCAG	AGTGTGTTC	CGTTAATTTT	
	humanEGR1	TTAAACGAA	AATAAAGTAG	CTGCGATTGG	G.....	.....	
		3801				3850	
40	mouseEGR1	.....	.....	.....	.....	.....	
	ratEGR1	GTAAATACTG	CTCGACTGTA	ACTCTCACAT	GTGACAAAAT	ACGGTTTGT	
	humanEGR1	.....	.....	.....	.....	.....	
		3851				3900	
45	mouseEGR1	.....	.....	.....	.....	.....	
	ratEGR1	TGGTTGGGTT	TTTTGTTGTT	TTTGAAAAAA	AAATTTTTTT	TTTGCCCGTC	
	humanEGR1	.....	.....	.....	.....	.....	
		3901				3950	
50	mouseEGR1	.....	.....	.....	.....	.....	
	ratEGR1	CCTTTGGTTT	CAAAAGTTTC	ACGTCTTGGT	GCCTTTGTGT	GACACACCTT	
	humanEGR1	.....	.....	.....	.....	.....	
		3951				4000	
55	mouseEGR1	.....	.....	.....	.....	.....	
	ratEGR1	GCCGATGGCT	GGACATGTGC	AATCGTGAGG	GGACACGCTC	ACCTCTAGCC	
	humanEGR1	.....	.....	.....	.....	.....	
		4001				4050	
60	mouseEGR1	.....	.....	.....	.....	.....	
	ratEGR1	TTAAGGGGGT	AGGAGTGATG	TTTCAGGGGA	GGCTTTAGAG	CACGATGAGG	

	humanEGR1	.....	.....	.....	.....	.....	.....
		4051					4100
5	mouseEGR1	.....	.....	.....	.....	.....	.....
	rateEGR1	AAGAGGGCTG	AGCTGAGCTT	TGGTTCTCCA	GAATGTAAGA	AGAAAAATTT	
	humanEGR1	.....	.....	.....	.....	.....	.....
		4101					4150
10	mouseEGR1	.....	.....	.....	.....	.....	.....
	rateEGR1	AAACAAAAA	TCTGAAGTCT	CAAAAGTCTA	TTTTTTTAAC	TGAAAAATGTA	
	humanEGR1	.....	.....	.....	.....	.....	.....
		4151					4200
15	mouseEGR1	.....	.....	.....	.....	.....	.....
	rateEGR1	GATTTATCCA	TGTTCCGGGAG	TTGGAATGCT	GCGGTTACCT	ACTGAGTAGG	
	humanEGR1	.....	.....	.....	.....	.....	.....
		4201					4250
20	mouseEGR1	.....	.....	.....	.....	.....	.....
	rateEGR1	CGGTGACTTT	TGTATGCTAT	GAACATGAAG	TTCATTATTT	TGTGGTTTTA	
	humanEGR1	.....	.....	.....	.....	.....	.....
		4251					4300
25	mouseEGR1	.....	.....	.....	.....	.....	.....
	rateEGR1	TTTTACTTCG	TACTTGTGTT	TGCTTAAACA	AAGTGACTTG	TTTGGCTTAT	
	humanEGR1	.....	.....	.....	.....	.....	.....
		4301					4350
30	mouseEGR1	.....	.....	.....	.....	.....	.....
	rateEGR1	AAACACATTG	AATGCGCTTT	ACTGCCCATG	GGATATGTGG	TGTGTATCCT	
	humanEGR1	.....	.....	.....	.....	.....	.....
		4351					4388
35	mouseEGR1	.....	.....	.....	.....	.....	.....
	rateEGR1	TCAGAAAAAT	TAAAAGGAAA	ATAAAGAAAC	TAACTGGT		
	humanEGR1	.....	.....	.....	.....	.....	.....

Example 1Characterisation of DNazymes ED5 and hED5Materials and Methods

5        *ODN synthesis.* DNazymes were synthesized commercially (Oligos Etc., Inc.) with an inverted T at the 3' position unless otherwise indicated. Substrates in cleavage reactions were synthesized with no such modification. Where indicated ODNs were 5'-end labeled with  $\gamma^{32}\text{P}$ -dATP and T4 polynucleotide kinase (New England Biolabs). Unincorporated label was  
10       separated from radiolabeled species by centrifugation on Chromaspin-10 columns (Clontech).

*In vitro transcript and cleavage experiments.* A  $^{32}\text{P}$ -labelled 206 nt NGFI-A RNA transcript was prepared by in vitro transcription (T3 polymerase) of plasmid construct pJDM8 (as described in Milbrandt, 1987,  
15       the entire contents of which are incorporated herein by reference) previously cut with *Bgl* II. Reactions were performed in a total volume of 20  $\mu\text{l}$  containing 10 mM  $\text{MgCl}_2$ , 5 mM Tris pH 7.5, 150 mM NaCl, 4.8 pmol of in vitro transcribed or synthetic RNA substrate and 60 pmol DNazyme (1:12.5 substrate to DNazyme ratio), unless otherwise indicated. Reactions were  
20       allowed to proceed at 37 °C for the times indicated and quenched by transferring an aliquot to tubes containing formamide loading buffer (Sambrook et al, 1989). Samples were run on 12% denaturing polyacrylamide gels and autoradiographed overnight at -80 °C.

*Culture conditions and DNazyme transfection.* Primary rat aortic SMCs  
25       were obtained from Cell Applications, Inc., and grown in Waymouth's medium, pH 7.4, containing 10% fetal bovine serum (FBS), 50  $\mu\text{g}/\text{ml}$  streptomycin and 50 IU/ml penicillin at 37 °C in a humidified atmosphere of 5%  $\text{CO}_2$ . SMCs were used in experiments between passages 3-7. Pup rat SMCs (WKY12-22 (as described in Lemire et al, 1994, the entire contents of  
30       which are incorporated herein by reference)) were grown under similar conditions. Subconfluent (60-70%) SMCs were incubated in serum-free medium (SFM) for 6 h prior to DNazyme (or antisense ODN, where indicated) transfection (0.1  $\mu\text{M}$ ) using Superfect in accordance with manufacturer's instructions (Qiagen). After 18 h, the cells were washed with  
35       phosphate-buffered saline (PBS), pH 7.4 prior to transfection a second time in 5% FBS.



*Northern blot analysis.* Total RNA was isolated using the TRIzol reagent (Life Technologies) and 25  $\mu$ g was resolved by electrophoresis prior to transfer to Hybond-N+ membranes (NEN-DuPont). Prehybridization, hybridization with  $\alpha^{32}$ P-dCTP-labeled Egr-1 or  $\beta$ -Actin cDNA, and washing was performed essentially as previously described (Khachigian et al, 1995).

*Western blot analysis.* Growth-quiescent SMCs in 100 mm plates (Nunc-InterMed) were transfected with ED5 or ED5SCR as above, and incubated with 5% FBS for 1 h. The cells were washed in cold PBS, pH 7.4, and extracted in 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 1% trasyolol, 10  $\mu$ g/ml leupeptin, 1% aprotinin and 2 mM PMSF. Twenty four  $\mu$ g protein samples were loaded onto 10% denaturing SDS-polyacrylamide gels and electroblotted onto PVDF nylon membranes (NEN-DuPont). Membranes were air dried prior to blocking with non-fat skim milk powder in PBS containing 0.05% (w:v) Tween 20. Membranes were incubated with rabbit antibodies to Egr-1 or Sp1 (Santa Cruz Biotechnology, Inc.) (1:1000) then with HRP-linked mouse anti-rabbit Ig secondary antiserum (1:2000). Where mouse monoclonal c-Fos (Santa Cruz Biotechnology, Inc.) was used, detection was achieved with HRP-linked rabbit anti-mouse Ig. Proteins were visualized by chemiluminescent detection (NEN-DuPont).

*Assays of cell proliferation.* Growth-quiescent SMCs in 96-well titer plates (Nunc-InterMed) were transfected with ED5 or ED5SCR as above, then exposed to 5% FBS at 37 °C for 72 h. The cells were rinsed with PBS, pH 7.4, trypsinized and the suspension was quantitated using an automated Coulter counter.

*Assessment of DNAzyme stability.* DNAzymes were 5'-end labeled with  $\gamma^{32}$ P-dATP and separated from free label by centrifugation. Radiolabeled DNAzymes were incubated in 5% FBS or serum-free medium at 37 °C for the times indicated. Aliquots of the reaction were quenched by transfer to tubes containing formamide loading buffer (Sambrook et al, 1989). Samples were applied to 12% denaturing polyacrylamide gels and autoradiographed overnight at -80 °C.

*SMC wounding assay.* Confluent growth-quiescent SMCs in chamber slides (Nunc-InterMed) were exposed to ED5 or ED5SCR for 18 h prior to a single scrape with a sterile toothpick. Cells were treated with mitomycin C (Sigma) (20  $\mu$ M) for 2 h prior to injury (Pitsch et al, 1996; Horodyski &

Powell, 1996). Seventy-two h after injury, the cells were washed with PBS, pH 7.4, fixed with formaldehyde then stained with hematoxylin-eosin.

*Rat arterial ligation model and analysis.* Adult male Sprague Dawley rats weighing 300-350 g were anaesthetised using ketamine (60 mg/kg, i.p.) and xylazine (8 mg/kg, i.p.). The right common carotid artery was exposed up to the carotid bifurcation via a midline neck incision. Size 6/0 non-absorbable suture was tied around the common carotid proximal to the bifurcation, ensuring cessation of blood flow distally. A 200  $\mu$ l solution at 4°C containing 500  $\mu$ g of DNAzyme (in DEPC-treated H<sub>2</sub>O), 30  $\mu$ l of transfecting agent and Pluronic gel P127 (BASF) was applied around the vessel in each group of 5 rats, extending proximally from the ligature for 12-15 mm. These agents did not inhibit the solidification of the gel at 37 °C. After 3 days, vehicle with or without 500  $\mu$ g of DNAzyme was administered a second time. Animals were sacrificed 18 days after ligation by lethal injection of phenobarbitone, and perfusion fixed using 10% (v:v) formaldehyde perfused at 120 mm Hg. Both carotids were then dissected free and placed in 10% formaldehyde, cut in 2 mm lengths and embedded in 3% (w:v) agarose prior to fixation in paraffin. Five  $\mu$ m sections were prepared at 250  $\mu$ m intervals along the vessel from the point of ligation and stained with hematoxylin and eosin. The neointimal and medial areas of 5 consecutive sections per rat were determined digitally using a customized software package (Magellan) (Halasz & Martin, 1984) and expressed as a mean ratio per group of 5 rats.

## Results and Discussion

The 7x7 nt arms flanking the 15 nt DNAzyme catalytic domain in the original DNAzyme design 7 were extended by 2 nts per arm for improved specificity (L.-Q. Sun, data not shown) (Figure 1). The 3' terminus of the molecule was capped with an inverted 3'-3'-linked thymidine (T) to confer resistance to 3'→5' exonuclease digestion. The sequence in both arms of ED5 was scrambled (SCR) without altering the catalytic domain to produce DNAzyme ED5SCR (Figure 1).

A synthetic RNA substrate comprised of 23 nts, matching nts 805 to 827 of NGFI-A mRNA (Figure 1) was used to determine whether ED5 had the capacity to cleave target RNA. ED5 cleaved the <sup>32</sup>P-5'-end labeled 23-mer within 10 min. The 12-mer product corresponds to the length between the

A(816)-U(817) junction and the 5' end of the substrate (Figure 1). In contrast, ED5SCR had no demonstrable effect on this synthetic substrate. Specific ED5 catalysis was further demonstrated by the inability of the human equivalent of this DNAzyme (hED5) to cleave the rat substrate over a wide range of stoichiometric ratios. Similar results were obtained using ED5SCR (data not shown). hED5 differs from the rat ED5 sequence by 3 of 18 nts in its hybridizing arms (Table 2). The catalytic effect of ED5 on a  $^{32}\text{P}$ -labeled 206 nt fragment of native NGFI-A mRNA prepared by *in vitro* transcription was then determined. The cleavage reaction produced two radiolabeled species of 163 and 43 nt length consistent with DNAzyme cleavage at the A(816)-U(817) junction. In other experiments, ED5 also cleaved a  $^{32}\text{P}$ -labeled NGFI-A transcript of 1960 nt length in a specific and time-dependent manner (data not shown).

Table 2. DNAzyme target sites in mRNA.

Similarity between the 18 nt arms of ED5 or hED5 and the mRNA of rat  
 5 NGFI-A or human EGR-1 (among other transcription factors) is expressed as a  
 percentage. The target sequence of ED5 in NGFI-A mRNA is 5'-807-A  
 CGU CCG GGA UGG CAG CCG-825-3' (SEQ ID NO: 13) (rat NGFI-A  
 sequence), and that of hED5 in EGR-1 is 5'-262-U CGU CCA GGA UGG CCG  
 CGG-280-3' (SEQ ID NO: 14) (Human EGR-1 sequence). Nucleotides in bold  
 10 indicate mismatches between rat and human sequences. Data obtained by a  
 gap best fit search in ANGIS using sequences derived from Genbank and  
 EMBL. Rat sequences for Sp1 and c-Fos have not been reported.

Gene	Accession number	Best homology over 18 nts (%)	
		ED5	hED5
Rat NGFI-A	M18416	100	84.2
20 Human EGR-1	X52541	84.2	100
Murine Sp1	AF022363	66.7	66.7
Human c-Fos	K00650	66.7	66.7
Murine c-Fos	X06769	61.1	66.7
Human Sp1	AF044026	38.9	28.9

25

To determine the effect of the DNAzymes on endogenous levels of  
 NGFI-A mRNA, growth-quiescent SMCs were exposed to ED5 prior to  
 stimulation with serum. Northern blot and densitometric analysis revealed  
 30 that ED5 (0.1  $\mu$ M) inhibited serum-inducible steady-state NGFI-A mRNA  
 levels by 55% (Figure 2a), whereas ED5SCR had no effect (Figure 2a). The  
 capacity of ED5 to inhibit NGFI-A synthesis at the level of protein was  
 assessed by Western blot analysis. Serum-induction of NGFI-A protein was  
 suppressed by ED5. In contrast, neither ED5SCR nor EDC, a DNAzyme  
 35 bearing an identical catalytic domain as ED5 and ED5SCR but flanked by  
 nonsense arms had any influence on the induction of NGFI-A (data not

shown). ED5 failed to affect levels of the constitutively expressed, structurally-related zinc-finger protein, Sp1. It was also unable to block serum-induction of the immediate-early gene product, c-Fos whose induction, like NGFI-A, is dependent upon serum response elements in its promoter and phosphorylation mediated by extracellular-signal regulated kinase (Treisman, 1990, 1994 and 1995; Gashler & Sukhatme, 1995). These findings, taken together, demonstrate the capacity of ED5 to inhibit production of NGFI-A mRNA and protein in a gene-specific and sequence-specific manner, consistent with the lack of significant homology between its target site in NGFI-A mRNA and other mRNA (Table 2).

The effect of ED5 on SMC replication was then determined. Growth-quiescent SMCs were incubated with DNase prior to exposure to serum and the assessment of cell numbers after 3 days. ED5 (0.1  $\mu$ M) inhibited SMC proliferation stimulated by serum by 70% (Figure 3a). In contrast, ED5SCR failed to influence SMC growth (Figure 3a). AS2, an antisense NGFI-A ODN able to inhibit SMC growth at 1  $\mu$ M failed to inhibit proliferation at the lower concentration (Figure 3a). Additional experiments revealed that ED5 also blocked serum-inducible  $^3$ H-thymidine incorporation into DNA (data not shown). ED5 inhibition was not a consequence of cell death since no change in morphology was observed, and the proportion of cells incorporating Trypan Blue in the presence of serum was not influenced by either DNase (Figure 3b).

Cultured SMCs derived from the aortae of 2 week-old rats (WKY12-22) are morphologically and phenotypically similar to SMCs derived from the neointima of balloon-injured rat arteries (Seifert et al, 1984; Majesky et al, 1992). The epitheloid appearance of both WKY12-22 cells and neointimal cells contrasts with the elongated, bipolar nature of SMCs derived from normal quiescent media (Majesky et al, 1988). WKY12-22 cells grow more rapidly than medial SMCs and overexpress a large number of growth-regulatory molecules (Lemire et al, 1994), such as NGFI-A (Rafaty & Khachigian, 1998), consistent with a "synthetic" phenotype (Majesky et al, 1992; Campbell & Campbell, 1985). ED5 attenuated serum-inducible WKY12-22 proliferation by approximately 75% (Figure 3c). ED5SCR had no inhibitory effect; surprisingly, it appeared to stimulate growth (Figure 3c). Trypan Blue exclusion revealed that DNase inhibition was not a consequence of cytotoxicity (data not shown).

To ensure that differences in the biological effects of ED5 and ED5SCR were not the consequence of dissimilar intracellular localization, both DNazymes were 5'-end labeled with fluorescein isothiocyanate (FITC) and incubated with SMCs. Fluorescence microscopy revealed that both FITC-ED5 and FITC-ED5SCR localized mainly within the nuclei. Punctate fluorescence in this cellular compartment was independent of DNzyme sequence. Fluorescence was also observed in the cytoplasm, albeit with less intensity. Cultures not exposed to DNzyme showed no evidence of autofluorescence.

Both molecules were 5'-end labeled with  $\gamma^{32}\text{P}$ -dATP and incubated in culture medium to ascertain whether cellular responsiveness to ED5 and ED5SCR was a consequence of differences in DNzyme stability. Both  $^{32}\text{P}$ -ED5 and  $^{32}\text{P}$ -ED5SCR remained intact even after 48 h. In contrast to  $^{32}\text{P}$ -ED5 bearing the 3' inverted T, degradation of  $^{32}\text{P}$ -ED5 bearing its 3' T in the correct orientation was observed as early as 1 h. Exposure to serum-free medium did not result in degradation of the molecule even after 48 h. These findings indicate that inverse orientation of the 3' base in the DNzyme protects the molecule from nucleolytic cleavage by components in serum.

Physical trauma imparted to SMCs in culture results in outward migration from the wound edge and proliferation in the denuded zone. We determined whether ED5 could modulate this response to injury by exposing growth-quiescent SMCs to either DNzyme and Mitomycin C, an inhibitor of proliferation (Pitsch et al, 1996; Horodyski & Powell, 1996) prior to scraping. Cultures in which DNzyme was absent repopulated the entire denuded zone within 3 days. ED5 inhibited this reparative response to injury and prevented additional growth in this area even after 6 days (data not shown). That ED5SCR had no effect in this system further demonstrates sequence-specific inhibition by ED5.

The effect of ED5 on neointima formation was investigated in a rat model. Complete ligation of the right common carotid artery proximal to the bifurcation results in migration of SMCs from the media to the intima where proliferation eventually leads to the formation of a neointima (Kumar & Lindner, 1997; Bhawan et al, 1977; Buck, 1961). Intimal thickening 18 days after ligation was inhibited 50% by ED5 (Figure 4). In contrast, neither its scrambled counterpart (Figure 4) nor the vehicle control (Figure 4) had any effect on neointima formation. These findings demonstrate the capacity of

ED5 to suppress SMC accumulation in the vascular lumen in a specific manner, and argue against inhibition as a mere consequence of a "mass effect" (Kitze et al, 1998; Tharlow et al, 1996).

Further experiments revealed the capacity of hED5 to cleave (human) EGR-1 RNA. hED5 cleaved its substrate in a dose-dependent manner over a wide range of stoichiometric ratios. hED5 also cleaved in a time-dependent manner, whereas hED5SCR, its scrambled counterpart, had no such catalytic property (data not shown).

The specific, growth-inhibitory properties of ED5 reported herein suggest that DNAzymes may be useful as therapeutic tools in the treatment of vascular disorders involving inappropriate SMC growth.

#### Example 2

##### Cleavage of human EGR-1 RNA by panel of candidate DNAzymes

15

To evaluate which specific DNAzymes targeting human EGR-1 (other than hED5) efficiently cleave EGR-1 RNA, we prepared *in vitro* transcribed 35S-labeled EGR-1 RNA and incubated this substrate with candidate DNAzymes for various times. The EGR-1 plasmid template (hs164) was prepared by subcloning bps 168-332 of human EGR-1 into pGEM-T-easy. A 388 nt 35S-labeled substrate was prepared by *in vitro* transcription using SP6 polymerase. Time-dependent cleavage of the substrate was tested using the following DNZzymes:

- 25 DzA: 5'-CAGGGGACAGGCTAGCTACAACGACGTTGCGGG-X-3' (SEQ ID NO: 15) ;  
DzB: 5'-TGCAGGGGAGGCTAGCTACAACGAACCGTTGCG-X-3' (SEQ ID NO: 16) ;  
DzC: 5'-CATCCTGGAGGCTAGCTACAACGAGAGCAGGCT-X-3' (SEQ ID NO: 17) ;  
30 DzE: 5'-TCAGCTGCAGGCTAGCTACAACGACTCGGCCTT-X-3' (SEQ ID NO: 18) ; and  
DzF: 5'-GCGGGGACAGGCTAGCTACAACGACAGCTGCAT-X-3' (SEQ ID NO: 19)  
35 where X denotes a 3'-3-linked T.

The DNAzyme that cleaved most effectively of this group was DzA, then DzB, then DzC. In contrast, DzE was inactive.

### Example 3

#### 5 Inhibition of induction of EGR-1 in human SMC by DzA

To determine whether DzA could block the induction of endogenous human EGR-1, we incubated growth-quiescent human aortic smooth muscle cells with 5% fetal bovine serum and observed the production of EGR-1  
10 protein by Western blot analysis. This band representing the EGR-1 protein was blocked by 0.5  $\mu$ M DzA, delivered using FuGENE6 (Roche Molecular Biochemicals) and unaffected by DzE. The blot was then stripped and reprobbed with antibodies to the transcription factor Spl. Results obtained showed that neither serum nor DzA affected induction of Sp1. A Coomassie  
15 Blue gel indicated that equal protein had been loaded.

The data demonstrate that DzA cleaves EGR-1 mRNA and blocks the induction of EGR-1 protein.

### Example 4

#### 20 Inhibition of human SMC proliferation by DzA

To ascertain whether proliferation of human SMCs could be inhibited by DzA, a population of SMCs was quantitated with and without exposure to DzA or DzE. SMC proliferation stimulated by 5% fetal bovine serum was  
25 significantly inhibited by 0.5  $\mu$ M DzA (Figure 5). In contrast, neither DzE nor ED5SCR had any effect (Figure 5). These data demonstrate that DzA inhibits human SMC proliferation.

### Example 5

#### 30 Inhibition of porcine SMC proliferation by DzA

The porcine and human EGR-1 sequences are remarkably well conserved (91%). Porcine retinal SMCs were used to determine whether DzA could block the growth of porcine SMCs. Our studies indicate that DzA (0.5  
35  $\mu$ M) could inhibit the proliferation of these cells (Figure 6). In contrast, DzE had no effect (Figure 6).



Example 6Delivery of DNAzyme into the porcine coronary artery wall

5 Porcine angioplasty and stenting are accepted models of human in-  
stent restenosis (Karas et al. 1992). The porcine coronary anatomy,  
dimensions and histological response to stenting are similar to the human  
(Muller et al. 1992). The Transport Catheter has previously been used to  
deliver antisense DNA targeting *c-myc* in humans (Serrys et al.  
10 1998) and the pig (Gunn & Cumberland, 1996) via the intraluminal route.  
Using this catheter, FITC-labeled DNAzyme was applied to the inner wall of  
a porcine coronary artery, *ex vivo*, from a newly explanted pig heart.  
DNAzyme (1000 µg) was delivered via the catheter in 2ml MilliQ H2O  
containing 300µl FuGENE6 and 1mM MgCl<sub>2</sub>. The FITC-labeled DNAzyme  
15 localised into the intimal cells of the vessel wall. These studies demonstrate  
that DNAzyme can be delivered to cells within the artery wall via an  
intraluminal catheter.

Throughout this specification the word "comprise", or variations such  
as "comprises" or "comprising", will be understood to imply the inclusion of a  
20 stated element, integer or step, or group of elements, integers or steps, but  
not the exclusion of any other element, integer or step, or group of elements,  
integers or steps.

It will be appreciated by persons skilled in the art that numerous  
variations and/or modifications may be made to the invention as shown in  
25 the specific embodiments without departing from the spirit or scope of the  
invention as broadly described. The present embodiments are, therefore, to  
be considered in all respects as illustrative and not restrictive. In addition,  
various documents are cited throughout this application. The disclosures of  
these documents are hereby incorporated by reference into this application to  
30 describe more fully the state of the art to which this invention pertains.

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30



Claims:

1. A DNzyme which specifically cleaves EGR-1 mRNA, the DNzyme  
5 comprising
  - (i) a catalytic domain which cleaves mRNA at a purine:pyrimidine cleavage site;
  - (ii) a first binding domain contiguous with the 5' end of the catalytic domain; and
  - 10 (iii) a second binding domain contiguous with the 3' end of the catalytic domain,wherein the binding domains are sufficiently complementary to the two regions immediately flanking a purine:pyrimidine cleavage site within the region of EGR-1 mRNA corresponding to nucleotides 168 to 332 as shown  
15 in SEQ ID NO:1, such that the DNzyme cleaves the EGR-1 mRNA.
2. A DNzyme as claimed in claim 1 wherein each binding domain is nine or more nucleotides in length.
- 20 3. A DNzyme as claimed in claim 1 or claim 2 in which the catalytic domain has the nucleotide sequence GGCTAGCTACAACGA (SEQ ID NO: 2).
4. A DNzyme as claimed in any one of claims 1 to 3 in which the cleavage site is selected from the group consisting of  
25
  - (i) the GU site corresponding to nucleotides 198-199;
  - (ii) the GU site corresponding to nucleotides 200-201;
  - (iii) the GU site corresponding to nucleotides 264-265;
  - (iv) the AU site corresponding to nucleotides 271-272;
  - (v) the AU site corresponding to nucleotides 301-302;
  - 30 (vi) the GU site corresponding to nucleotides 303-304; and
  - (vii) the AU site corresponding to nucleotides 316-317.
5. A DNzyme as claimed in claim 4 in which the cleavage site is the AU site corresponding to nucleotides 271-272.

6. A DNAzyme as claimed in claim 1 which has a sequence selected from the group consisting of:

- 5 (i) 5'-caggggacaGGCTAGCTACAACGAcgttgcg (SEQ ID NO: 3);
- (ii) 5'-tgcaggggaGGCTAGCTACAACGAaccgttgcg (SEQ ID NO: 4);
- (iii) 5'-catcctggaGGCTAGCTACAACGAgagcaggct (SEQ ID NO: 5);
- (iv) 5'-ccgcgccaGGCTAGCTACAACGAcctggacga (SEQ ID NO: 6);
- (v) 5'-ccgctgccaGGCTAGCTACAACGAcccgacgt (SEQ ID NO: 7);
- 10 (vi) 5'-gcggggacaGGCTAGCTACAACGAcagctgcat (SEQ ID NO: 8);
- (vii) 5'-cagcggggaGGCTAGCTACAACGAatcagctgc (SEQ ID NO: 9); and
- (viii) 5'-ggtcagagaGGCTAGCTACAACGActgcagcgg (SEQ ID NO: 10).

15 7. A DNAzyme as claimed in claim 6 which has the sequence:  
5'-ccgcgccaGGCTAGCTACAACGAcctggacga (SEQ ID NO: 6).

8. A DNAzyme as claimed in any one of claims 1 to 7, wherein the 3'-end nucleotide residue is inverted in the binding domain contiguous with the 3' end of the catalytic domain.

9. A pharmaceutical composition comprising a DNAzyme according to any one of claims 1 to 8 and a pharmaceutically acceptable carrier.

25 10. A method of inhibiting EGR-1 activity in cells which comprises exposing the cells to a DNAzyme according to any one of claims 1 to 8.

11. A method of inhibiting proliferation or migration of cells in a subject which comprises administering to the subject a prophylactically effective dose of the pharmaceutical composition according to claim 9.

12. A method of treating a condition associated with cell proliferation or migration in a subject which comprises administering to the subject a therapeutically effective dose of the pharmaceutical composition according to claim 9.

13. A method as claimed in any one of claims 10 to 12 wherein the cells are vascular cells.

5 14. A method as claimed in any one of claims 10 to 12 wherein the cells are cells involved in neoplasia.

15. A method as claimed in claim 12 wherein the condition associated with cell proliferation or migration is selected from the group consisting of  
10 post-angioplasty restenosis, vein graft failure, hypertension, transplant coronary disease and complications associated with atherosclerosis or peripheral vascular disease.

16. An angioplastic stent for inhibiting the onset of restenosis, which  
15 comprises an angioplastic stent operably coated with a prophylactically effective dose of a DNAzyme according to any one of claims 1 to 8.

17. A method for inhibiting the onset of restenosis in a subject undergoing angioplasty, which comprises topically administering a prophylactically  
20 effective dose of a pharmaceutical composition according to claim 9 to the subject at around the time of the angioplasty.

18. A method according to claim 17 in which the pharmaceutical composition is administered by catheter.

25

19. A method for inhibiting the onset of restenosis in a subject undergoing angioplasty, which comprises topically administering a stent according to claim 15 to the subject at around the time of the angioplasty.

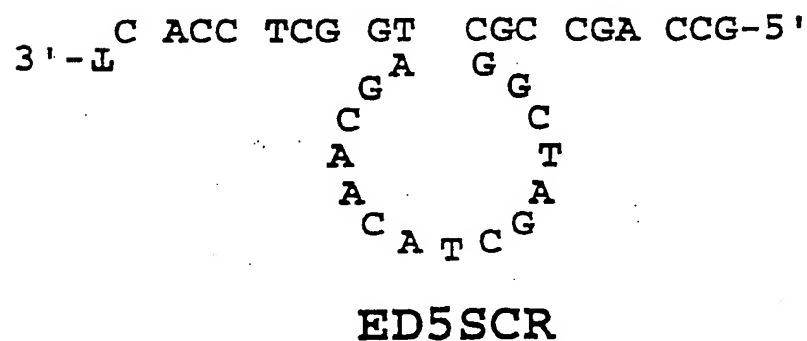
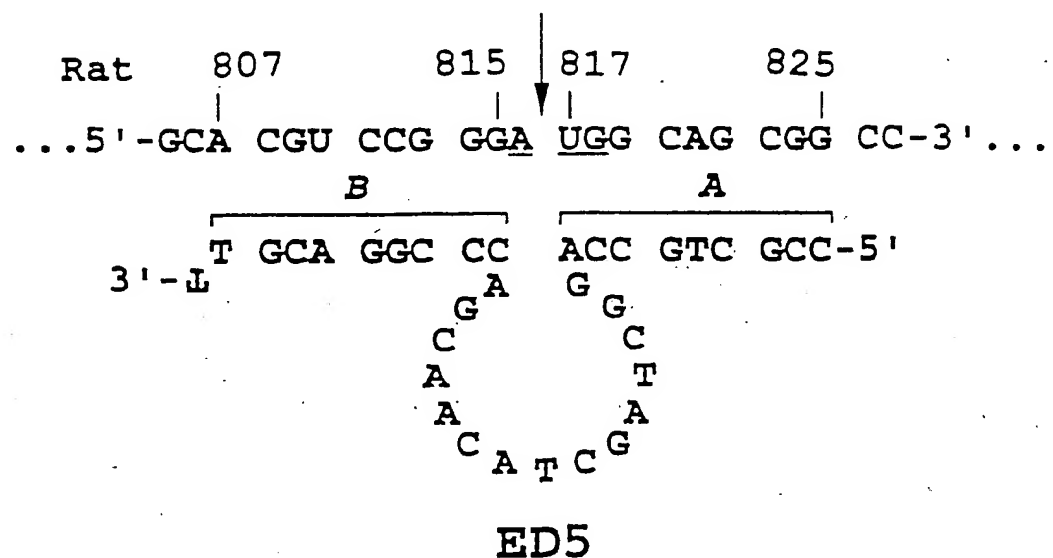


Figure 1

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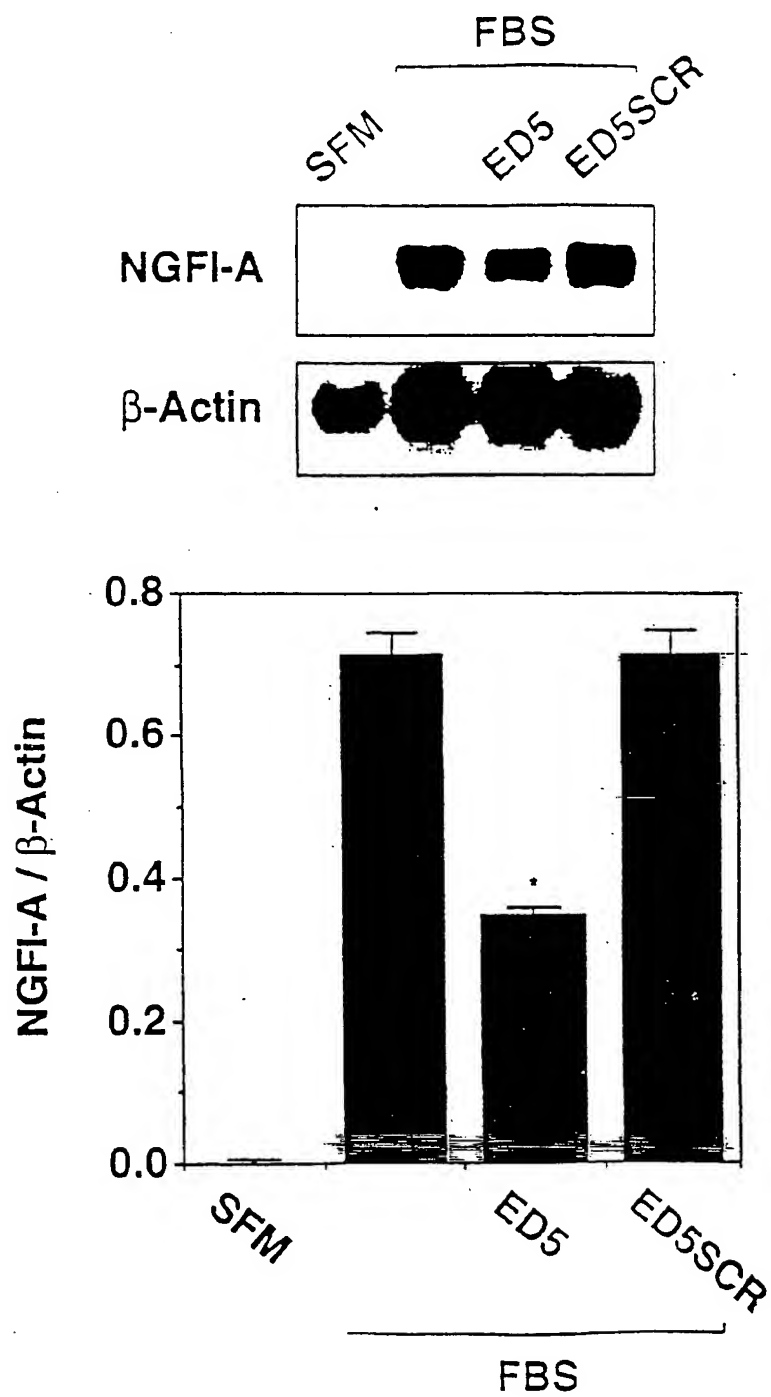


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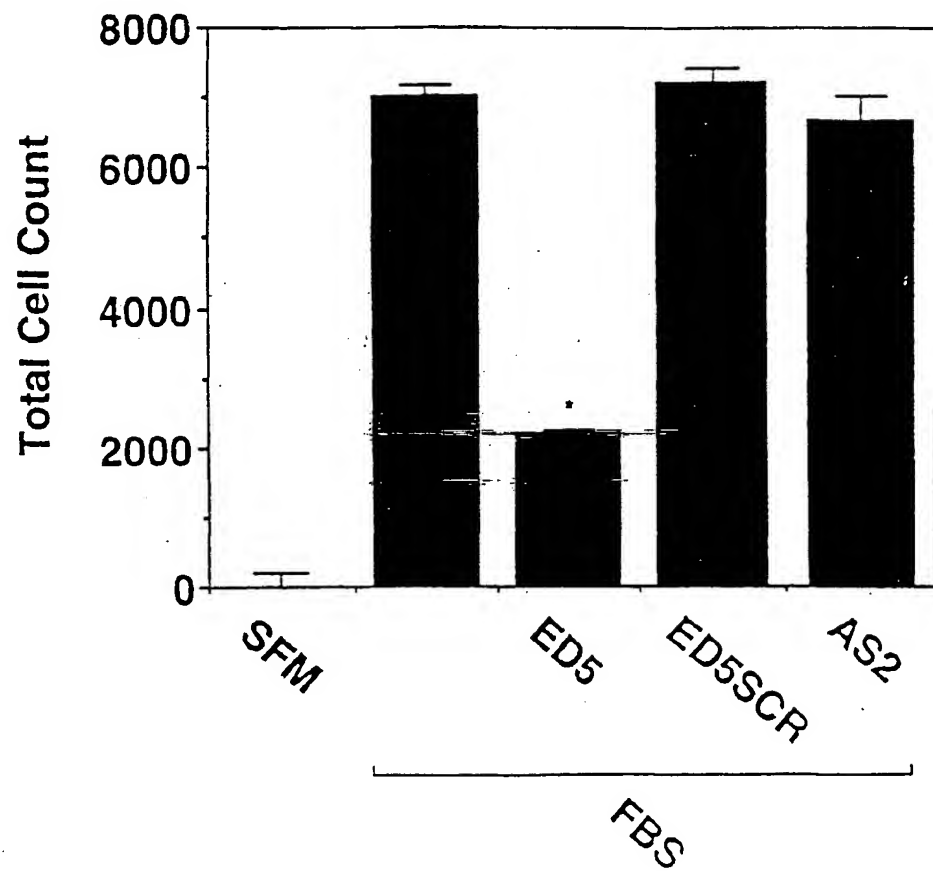


Figure 3A

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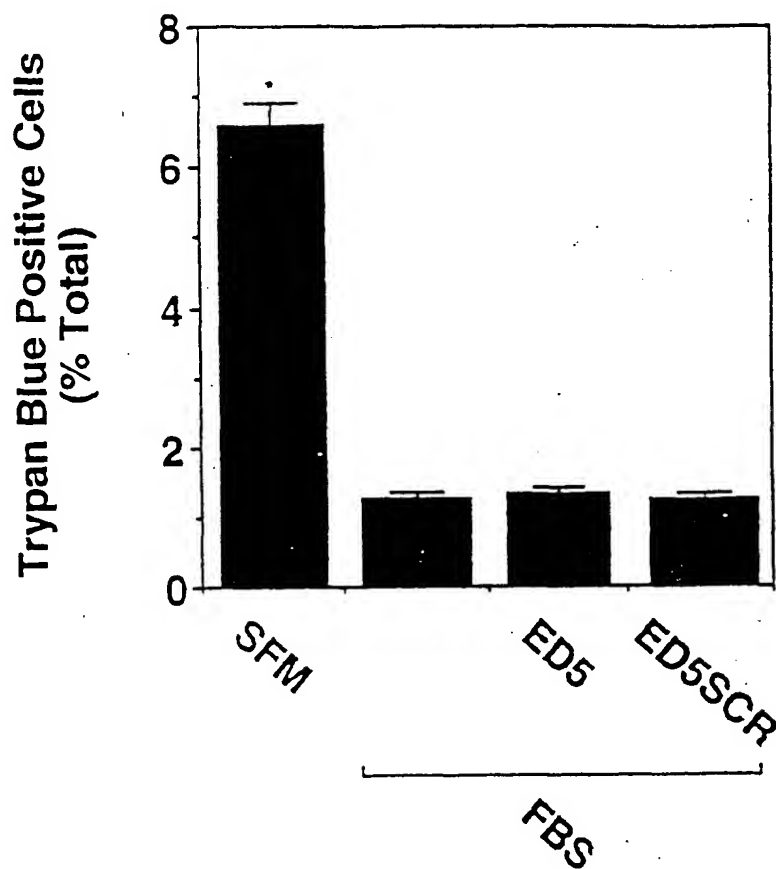


Figure 3B

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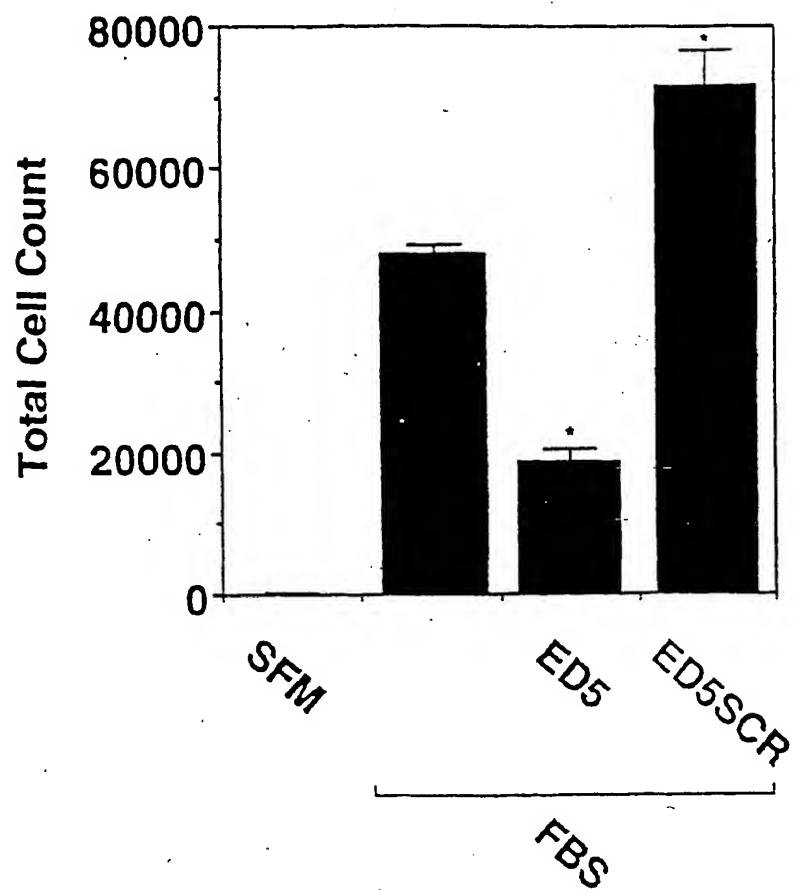


Figure 3C



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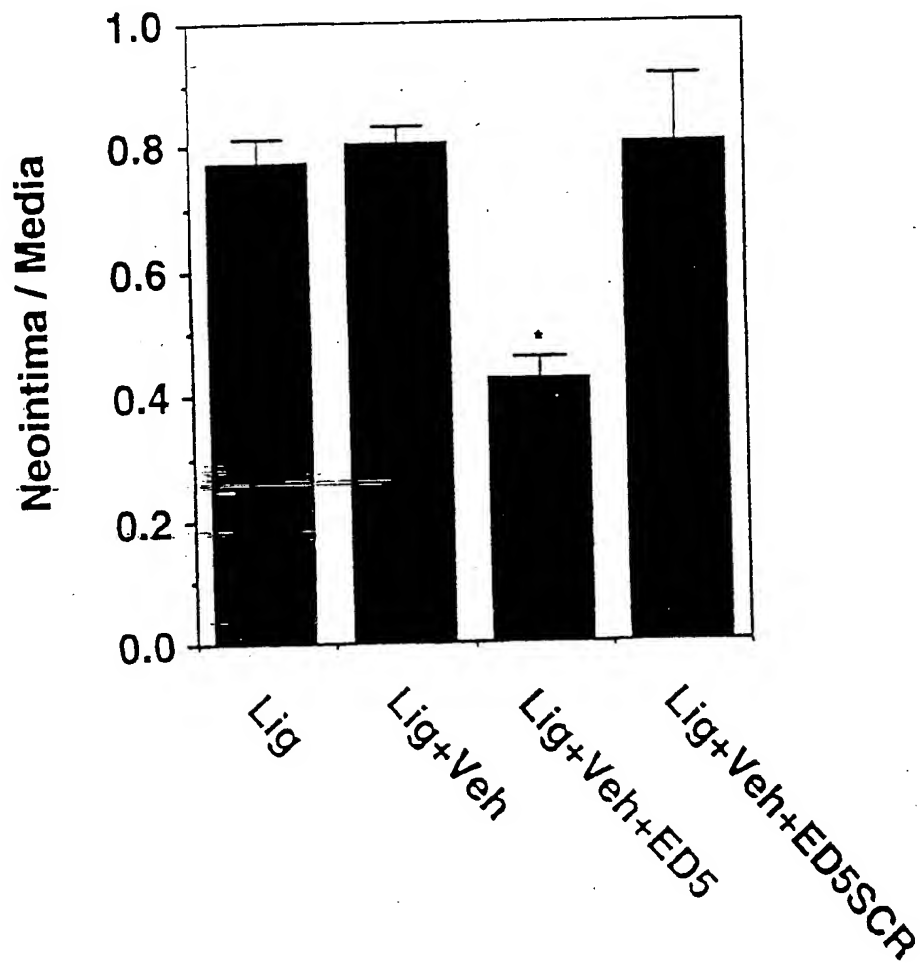


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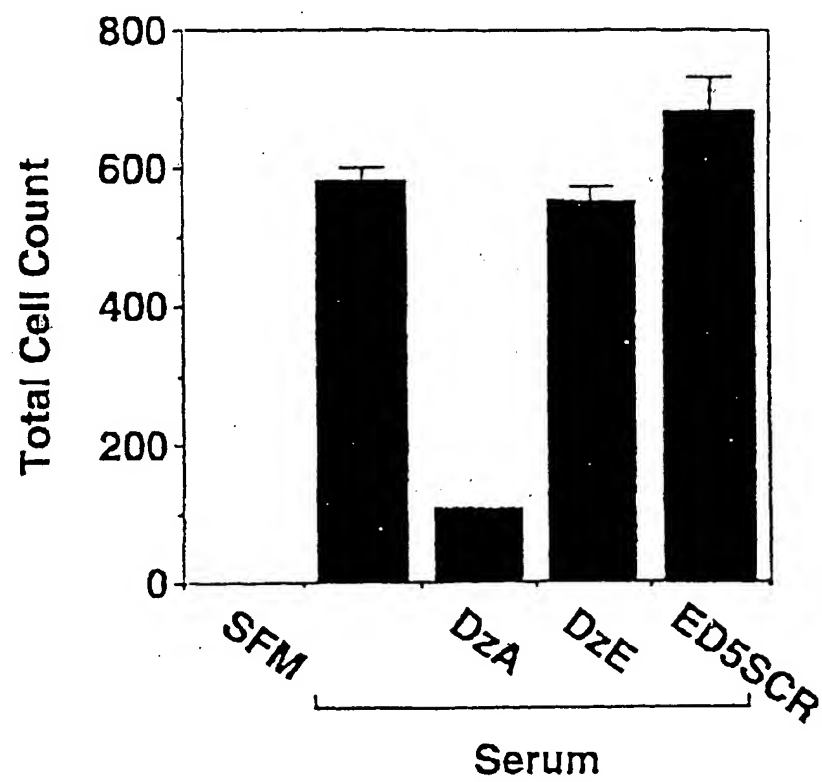


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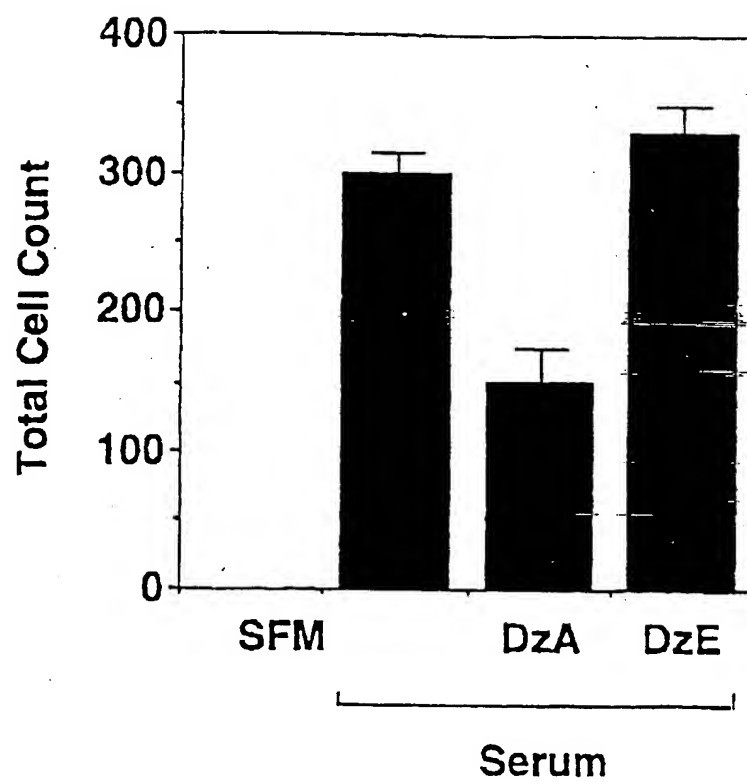


Figure 6

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/AU00/00011

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
Int. Cl. <sup>7</sup> : C12N 9/16; A61K 38/46; A61I 27/34, 33/12; C12Q 1/68.		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols) World Patent Index and Chemical Abstracts. Keywords: See electronic database box below.		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Medline, Biological Abstracts and Biotechnology Abstracts. Keywords: See electronic database box below. EMBL, Genebank, DDBJ, PDB and Dgene(Derwent sequence database)		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) (DNAzyme# or deoxyribozyme# or DNA(enzyme# or catalytic(10n)DNA) and (EGR()1 or early()growth()response# or ngf? or zif268 or tis8 or krox24 or cef5 or cet5). Nucleic acid Sequence SEQ ID NO:2		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	Santiago F S <i>et al</i> "New DNA enzyme targeting Egr-1 mRNA inhibits vascular smooth muscle proliferation and regrowth after injury." Nature Medicine volume 5 number 11 November 1999 pages 1264-1269. See the whole article especially figure 1.	1-15
X,Y	WO 97/32979 (UNISEARCH LIMITED) 12 September 1997. See whole document especially pages 4-6 and claims.	1-15
PY	Caims M. J. <i>et al</i> "Target site selection for an RNA-cleaving catalytic DNA" Nature Biotechnology vol 17, May 1999 pages 480-486. See the whole document. Category Y with document D2.	1-8
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex		
<p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier application or patent but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p>		
Date of the actual completion of the international search 3 March 2000		Date of mailing of the international search report - 9 MAR 2000
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaaustralia.gov.au Facsimile No. (02) 6285 3929		Authorized officer  J.H. CHAN Telephone No.: (02) 6283 2340

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU00/00011

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, Y	WO 98/49346 (THE SCRIPPS RESEARCH INSTITUTE) 5 November 1998.. See the whole document, especially pages 95-98, sequence id no. 85 and figures 8-10. Category Y with document D2.	1-8. 9-15.
X, Y	WO 96/17086 (THE SCRIPPS RESEARCH INSTITUTE) 6 June 1996. See the whole document, especially pages 11, 12, 32, 51-54, sequence id no. 85 and figures 8 and 9. Category Y with document D2.	1-8. 9-15.
X, Y	Santoro S W and Joyce G F "A general purpose RNA-cleaving DNA enzyme" Proc Natl Acad Sci USA volume 94 pages 4262-4266 April 1997. See the whole document, especially pp 4264-6 and figure 2. Category Y with document D2.	1-8. 9-15.
X, Y	Santoro S W and Joyce G F "Mechanism and utility of an RNA-cleaving DNA enzyme" Biochemistry 1998 September 22, 37, 13330-42 See the whole document especially pages 13331, 13337-41 and figure 1. Category Y with document D2.	1-8. 9-15
P, Y	WO 99/50452 (JOHNSON & JOHNSON RESEARCH PTY LIMITED) 7 October 1999. See the whole document, especially pages 17-25. Category Y with document D2.	1-8.
Y	Genebank accession no. X52541 Publication date 12 September 1993. Category Y with document D2.	1-8.
Y	Genebank accession no. X52541 Publication date 12 September 1993. Category Y with document D2.	1-8.

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

International application No.  
**PCT/AU00/00011**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report				Patent Family Member			
WO	97/32979	AU	20865/97	CA	2248350	EP	934404
WO	98/49346	AU	72675/98	EP	981646		
WO	96/17086	AU	45950/96	BR	9510003	CA	2205382
		CN	1173207	EP	792375	FI	972333
		HU	77576	NO	972483	US	5807718
WO	99/50452	AU	35303/99				
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